INCIDENCE OF THE PRESENCE OF PLASTICS ON EARTHWORMS: EFFECTS ON VERMICOMPOSTING PROCESSES

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INFORMAN:

Que D. Zbigniew Emil Blesa Marco ha realizado bajo nuestra supervisión el trabajo titulado “Incidence of the presence of plastics on earthworms: effects on vermicomposting processes”, conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Lo que firmamos para los efectos oportunos, en Orihuela a …… de enero de 2024

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RESUMEN

En todo el mundo se producen anualmente unos 360 millones de toneladas de plástico, de las cuales en torno al 40% se consume en el sector de envasado de alimentos y el 3,5% directamente en agricultura. En la actualidad, sólo se recicla algo más del 30% de los residuos plásticos generados en la UE, mientras que el resto se incinera, se deposita en vertederos o se libera al medio ambiente. La gestión de estos plásticos al final de su vida útil supone un reto medioambiental en el sector agroalimentario, ya que es frecuente su recuperación incompleta tras la temporada de cultivo, lo cual conduce a la acumulación de restos de plásticos en los residuos orgánicos. A pesar de ello, el efecto de estos restos plásticos en los residuos orgánicos sometidos a diferentes biotratamientos ha sido poco estudiado. Uno de los biotratamientos más adecuados para generar y transformar residuos orgánicos de forma sostenible es el vermicompostaje. Está técnica de bajo coste es empleada para producir actualmente biofertilizantes, siendo la presencia de plástico en los flujos orgánicos a circularizar un problema creciente.

El objetivo de esta tesis doctoral fue estudiar el efecto que causaba la presencia de agroplásticos al proceso de vermicompostaje, utilizando lombrices de tierra de la especie *Eisenia fetida*. Primero se realizó una caracterización de los plásticos más empleados en la Agroindustria y se propusieron diferentes pretratamientos abióticos (térmicos, fotoxidación, químicos y técnica e-beam) de los plásticos, que simulaban su degradación o envejecimiento en el medioambiente. Posteriormente se ensayó el efecto que causaba la presencia de agroplásticos en un proceso de vermicompostaje a escala microcosmos. Se determinó tanto el efecto que causaba a nivel fisiológico en los individuos de *E. fetida* (cambios morfológicos y de estrés oxidativo), así como los cambios en las características y calidad del biofertilizante obtenido. También se identificó, extrajo y produjo consorcios microbianos que vivían en este tipo de ambientes contaminados. Se utilizan consorcios microbianos con capacidad de degradar polímeros (EXO-PMC) así como los procedentes del tracto digestivo de *E. fetida* expuesta a agroplásticos (ENDO-PMC) con capacidad probiótica. Por último, se desarrolló un ensayo de exposición a agroplásticos mediante un proceso de vermicompostaje a escala de mesocosmos, pero con la adición de consorcios EXO and ENDO-PMC para comprobar si estos microrganismos beneficiosos eran capaces de fortificar y mejorar las capacidades de las lombrices de tierra y del proceso, en presencia de agroplásticos.

Los resultados obtenidos mostraban como los agroplásticos sometidos a los diferentes pretratamientos realizados presentaban cambios estructurales, con aparición de grupos carboxilo e introducción de oxígeno en su cadena. En los ensayos de exposición, los agroplásticos causaron un efecto negativo sobre *E. fetida* a diferentes niveles, con pérdida de peso corporal, síntomas de estrés oxidativo y/o neurotoxicidad e incluso mortalidad de algunos individuos a más largo plazo, además se detectaron cambios en la calidad y el contenido NPK del vermicompost obtenido (destacando el LDPE + LLDPE, PET y PS). La aplicación de los PMC, produjeron una mejora significativa en las capacidades de *E. fetida* para soportar el efecto negativo que causa la presencia de agroplásticos y se obtuvieron biofertilizantes de mayor calidad.

Abstract

Worldwide, approximately 360 million tonnes of plastic are produced annually, with approximately 40% used in the food packaging sector and 3.5% in the agricultural sector. Nowadays, just over 30% of the plastic waste generated in the European Union is recycled, while the remainder end up in landfills, is either incinerated, or released into the environment. Their end-of-life management constitutes an environmental challenge in agri-food sector due to usual incomplete recovery after the crop season, with the subsequent accumulation of plastic waste in organic waste. Despite this, the impact of these plastic debris in organic waste subjected to various biotreatments remain unexplored. One of the most suitable biotreatments for valorization and stabilization of organic waste is vermicomposting process. This cost-effective technique is increasingly used to design and produce bio-based fertilizers.

The aim of this doctoral thesis was to investigate the potential impact induced from the presence of Agri-food waste plastics (AWP) in the vermicomposting process, carried out by earthworms of the species *Eisenia fetida*. Initially, a characterization of the most commonly used plastics in the agri-food sector was conducted, and several abiotic pre-treatments (thermal, photo-oxidation, chemical, and e-beam) for the plastics were carried out in order to simulate their degradation or aging in the environment. Subsequently, the impact derived from the presence of AWP in a vermicomposting process at the microcosm scale also was examined. The study assessed the physiological effects on *E. fetida* individuals, including morphological changes and biomarkers of oxidative stress, as well as the change in the characteristics and quality of the biofertilizer obtained. Microbial consortia were identified, isolated, and cultured from contaminated environments. Microbial consortia with polymer-degrading capabilities (EXO-PMC) and from the gut-microbiome of *E. fetida* exposed to AWP (ENDO-PMC) with probiotic capacities were used. Subsequently, an AWP exposure bioassay was developed using a mesocosm-scale vermicomposting process, inoculated with EXO and ENDO-PMC to assess the performance of beneficial microorganisms in producing fortified earthworms, improve their capabilities, and enhance the process in the presence of AWP.

The results obtained revealed structural changes in AWP subjected to several pre-treatments, including the increasing presence of carboxyl groups and the introduction of more oxygen atoms into the polymer chain. In the exposure bioassay, the presence of AWP induced negative effects on *E. fetida* as evidenced the body weight loss, oxidative stress and/or neurotoxicity, and even mortality of some individuals in long-term experiments. Additionally, changes were observed in the quality and NPK content of the vermicompost obtained, with notable effects on LDPE + LLDPE, PET, and PS. The application of PMCs resulted in a significant improvement in the ability of *E. fetida* to alleviated the negative effects caused by the presence of AWP, leading to the production of higher quality biofertilizers.

INDEX

1. Introduction

1.1. Used and management of plastics in Europe

Europe's total demand for plastic has risen to 49 million tonnes per year, of which 37-38 % is used for packaging. Packaging plastics consumed worldwide accounts for 35 % (Al-Salem et al., 2009). Around 60 % of all plastic packaging is used for food and beverages, while the rest covers non-food applications, such as healthcare, cosmetics, consumer, household, apparel, and shipment packaging (Plastics Europe, 2020). More specifically in the municipal solid waste (MSW) stream, the various sources of plastics include domestic items (empty food containers, packaging foam, disposable cups, plates, cutlery, etc.) and agricultural items (mulch films, feed bags, fertilizer bags, etc.). By far the largest share of all post-consumer plastics in MSW is packaging waste. Packaging products are ubiquitous and tend to have short lifespans (Scarascia-Mugnozza, 1994). It's estimated that roughly 40 % of plastic products have a service life of less than one month (Achilias et al., 2007). The five polymers most applied in plastic packaging include PET, PE, PP, PS, and PVC (Groh et al., 2019; Villanueva and Eder, 2014).

On average in the EU, 31 kg of plastic packaging waste is produced per person per year (Eurostat, 2018). The MSW contains usually a mixture of plastics, and this amalgam is combined with organic or other residual household wastes. Plastic packaging is diverse and made of multiple polymers and numerous additives, along with other components, such as adhesives or coatings for different specifications to meet the manufacturer functional and/or aesthetic requirements. Further, packaging can contain residues from substances used during manufacturing, such as solvents, along with non-intentionally added substances (NIAS), such as impurities, oligomers, or degradation products. In addition, the chemical identity and quantity of many non-intentionally added substances present in finished plastic packaging are rarely addressed and often remain unknown (MIT, 2018). This diversity complicates the recycling process in cost and the quality of the recycled plastic (European Commission, 2018). Recycling rates of plastic packaging waste in Europe range between 26 and 52% and this wide range can be explained by differences in collection schemes, available infrastructure, and consumer behaviour. On average, the 40% recycling of plastic packaging rate represents an increase compared to 2016, thanks to improved waste collection (Groh et al., 2019). Much plastic waste generated today is not properly collected, recovered, or disposed of, contributing to the increasing accumulation of plastic wastes.

Separate collection of plastic waste for recycling includes in-house bin systems, kerbside collection, drop-off containers and recycling centres. Recycling centres are large drop-

off points where private citizens (free of charge) bring and ‘‘source separate” household waste into many waste fractions and represent an important yet often overlooked collection method in some European countries, e.g., UK, Denmark, Sweden and Norway (MIT, 2018). In Europe, landfilling and incineration are the dominant approaches to manage plastic waste, at 31% and 39%, respectively (Plastics Europe, 2020). Less than 30% of post-consumer plastic waste is collected for recycling, most of which is either exported out of Europe or destined for low value applications. The exported plastic waste (3.05 million tonnes in 2015) is mostly transported to Asian countries (Velis, 2014; MIT, 2018). However, with China’s ban on all plastic waste imports from 2018, Europe will need to better manage its plastics at local level, as well as changing the ways we produce and consume plastics in all sectors of the economy (Schweitzer et al., 2018). Plastics from MSW must be effectively treated through different valorization methods, including preferentially first the in-situ recycling, mechanical recycling, thermolysis, chemo-lysis and the last one, the energy recovery.

1.2. Properties and environmental concerns related with the use of agroplastics

One of the great challenges facing agriculture and the food industry is the use of plastics. It is a very complex management to carry it out and the rate of recovery of plastic waste for agricultural use is very low due to the difficulty in recycling this type of materials. Since 1950, plastic production has increased on an average of almost 10 % every year on a global basis (Scarascia-Mugnozza et al., 2011). The great diffusion of plastic in our society is due to a range of characteristics of the plastic material like their low-density, lightness, strength, workability and low cost compared to other materials. However, such diverse consumption leads to a diverse waste stream (European Commission, 2018). The most common polymers used in agriculture are polyethylene (PE) and polypropylene (PP) (Sica et al., 2008; Scarascia-Mugnozza et al., 2011). Concerning food packaging plastic waste, the 5 most common polymers used are listed by several authors as PE (including LDPE, LLDPE, and HDPE), PP, PS, PET and PVC.

Of the total plastic waste, 60% corresponds to packaging and 5% corresponds to agricultural plastic waste. While landfill has decreased over the past decade thanks to landfill restrictions of recyclable and recoverable waste, incineration has grown. So, 31% of plastic waste ends up in landfills and 39% is incinerated (Scarascia-Mugnozza et al., 2011; European Commission, 2018). In general, the most used recycling strategies are

mechanical, chemical recycling or energy recovery. Depending on the product to be recycled this can result in high costs. In addition, during their use for application such as those of food packaging and those in agriculture, plastic materials are contaminated by different impurities like food, soil and agrochemical particles difficult to be removed, washed away, from the post consume plastic. All four levels of plastic recycling processes primary and secondary mechanical recycling, chemical depolymerisation and thermal recovery result in environmental and human pollution. To sum up, it should be noted that the use, sorting, recovery and recycling of plastic waste remains largely unresolved (European Commission, 2018).

Plastic materials are widely used in European agriculture because they contribute to increase the quality and the quantity of production. The world consumption of plastics in agriculture amounts yearly to 6.5 million tons. Some of the reported benefits of using plastic materials in agricultural fields result from increased yields, earlier harvests, reduction of herbicide and pesticide consumption, frost protection and water conservation, without leaving out the importance of plastic material to preserve, transport, package and commercialize agri-food products. It also provided a more efficient use of farmland, higher quality of crops and a resultant healthier environment. Furthermore, plastics-based agricultural systems provide effective solutions for crop growing in many ways: in arid regions, for example, plastic irrigation tapes, piping and drainage systems can cut irrigation costs by one to two-thirds, while as much as doubling crop yield. The market of plastics used for these purposes in Europe involves hundreds of thousands of hectares and thousands of tons of plastic films per year. Table 1 gives a summary characterization of the most used plastics in agriculture for each use or function they perform.

About twenty distinct groups of plastics for agricultural use exist, each with various formulations available to enable the best choice for each specific application. To produce flexible, semi-rigid and/or rigid materials, the main polymers used in agriculture are the PE and the PP. PE has a wide range of agricultural uses because of its low cost, good workability, high impact resistance, excellent chemical resistance and electrical insulation properties. LDPE and linear LDPE (LLDPE) are mainly used to produce films (for greenhouses, low tunnels, mulching, and silage), due to its high tear and impact strength (Dorigato et al., 2011). In addition, LDPE and LLDPE are strong and flexible and have good transparency, moisture and gas barrier properties (Niaounakis and Kontou, 2005). The long chain branching and the narrower molecular weight distribution of LDPE

determine its greater transparency, gloss and better processability. LLDPE has greater tensile and impact strength, better heat-seal properties, and lower cost (Chiappero et al., 2021), which makes this polymer the most versatile type with the widest range of film applications (Al-Salem et al., 2009). PP has a lower impact strength, but superior working temperature and tensile strength. PP is most widely used as fibres and filaments produced by extrusion and is used in agriculture for piping, sheeting, nets, and twines (McEwan, 2002).

Table 1. Characterization of the most used agroplastics.

Applications	Basic composition	Additives
Greenhouse films	LDPE, LDPE.IR, EVA, LLDPE	Anti-fog, Photo-selective, UV stabilizers, Long Infra-red properties enhancer master-batch
Low tunnel films	LDPE, EVA, LLDPE, PVC	UV stabilizers, Infra-red properties enhancer
Mulching films	LDPE transparent or black	Coloured pigments, UV stabilizers, Carbon black
Covering vineyard, orchards	LDPE, EVA	UV stabilizers, Coloured pigments
Nonwoven / Floating covers	PP, LDPE perforated	-
Nets for collecting	PP, HDPE	Coloured pigments
Woven nets (hail, wind, bird, shade)	HDPE	Coloured pigments
Silage films and protective covering	LDPE	Coloured pigments
Irrigation and drainage	LDPE,HDPE, PVC, PRFV	Coloured pigments
Other (rigid shhets, pots, twine, etc)	LDPE, PP, PS, HDPE, PRFV, PVC, PMMA	Coloured pigments
Pesticides cans	LDPE, HDPE	Coloured pigments
Fertilizers bags	LDPE, HDPE	Coloured pigments

Agricultural plastics, produced with fossil raw resources, are subjected to degradation due to their exposure to atmospheric agents such as solar radiation, wind, rain, hail, air temperature and humidity, to the installation and utilization conditions, and to the pesticides used during the cultivation period. Plastic degradation results in the production of huge and increasing amounts of plastic waste when the plastics are dismantled (Scarascia-Mugnozza, 1994; Sica et al., 2008), henceforth referred to as agri-food waste plastic (AWP). Most of AWP come from films and, in the Southern European countries, these films come from protected cultivations (greenhouses, low tunnels, mulching and covering) while in the North European countries they come from silage films and wraps.

The existing technological solutions to process AWP are mainly incineration, gasification and pyrolysis. Unfortunately, abandonment and burning are practices still frequently used both in Italy and in other countries, although they are against the law.

1.3. Biotic and abiotic degradation routes of agroplastics

The American Society for Testing and Materials (ASTM) and the International Organization for Standardization (ISO) define degradation as "an irreversible process leading to a significant change of the structure of a material, typically characterized by a loss of properties (e.g., integrity, molecular weight, structure, or mechanical strength) and/or fragmentation. Degradation is affected by environmental conditions and proceeds over a period comprising one or more steps" (ISO, 1993; ASTM, 1991). In a polymer, any physical or chemical change is a result of environmental factors, such as light, heat, moisture, chemical conditions, or biological activity. Thus, the processes inducing changes in polymer properties (deterioration of functionality) due to chemical, physical, or biological reactions resulting in bond scission and subsequent chemical transformations (formation of structural in homogeneities) are considered as polymer degradation (Ali et al., 2008).

The process of plastic degradation is determined by both environmental conditions and physicochemical properties of polymeric substances. The physicochemical properties of plastic play an important role in the degradation process. Thus, the most widely used plastics can be classified into two main categories with different resistance to degradation: C-C backbone polymers, such as PE, PVC, PS and PP, and heteroatomic polymers, including PET and PU (Ali et al., 2021). C-C backbone polymers are resistant to hydrolysis and biodegradation and susceptible to thermal oxidation (Krueger et al., 2015), while heteroatomic polymers may be processed through photo-oxidation, hydrolysis, and biological degradation (Gewert et al., 2015). In Figure 1 is shown the market shares of the most significant plastics, also including their hydrolizable capacity (Inderthal et al., 2020).

Plastic susceptibility to abiotic and biotic degradation depends on backbone composition and chain length, with long carbon chain such as PP, making polymers resistant to degradation (Huerta Lwanga et al., 2016; Fotopoulou and Karapanagioti, 2017). However, the incorporation of heteroatoms, such as in PET and PU (oxygen-containing polymers) constitutes plastic susceptible to biodegradation and thermal degradation

(Singh and Sharma, 2008). Moreover, the polymer hydrophobicity affects the degradation efficiency, where the degradation rate increases with increasing hydrophilicity (Padsalgikar, 2017).

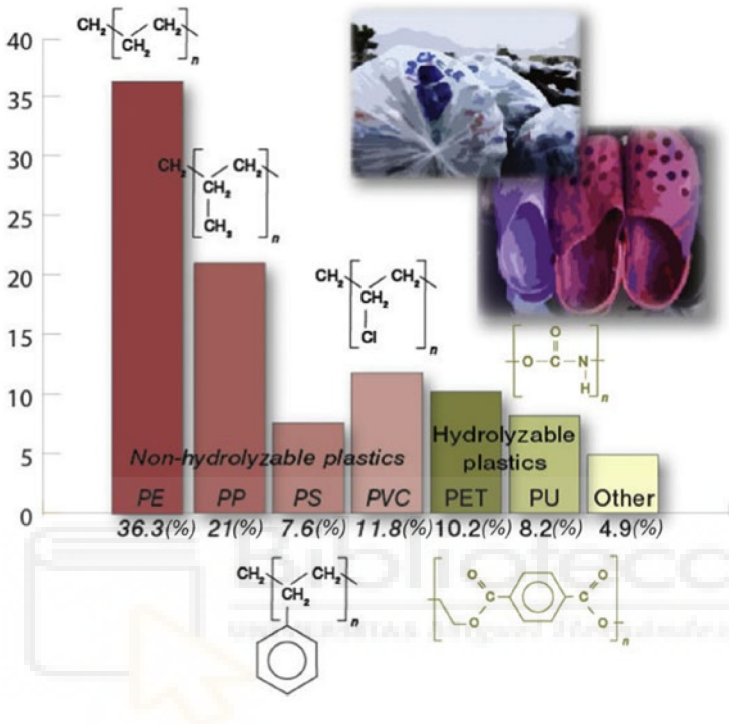


Figure 1. Market shares of the six commercially most important plastic material types (Inderthal et al. 2020). Their monomer structures in high molecular weight polymer chains are shown adjacent to bars.

In addition, the degradation rate depends on the polymer crystallinity (Jenkins and Harrison, 2008). In this sense, a generally accepted hypothesis is the “chain-flexibility hypothesis” (Figure 2), based on the idea that the reaction rates vary with the flexibility or mobility of molecular chains in the substrate. When a polymer had more free volume, caused by lower crystallinity, a higher amount of branching, domains that are more mobile, the addition of plasticizers, or higher experimental temperatures, then the molecular chains have more flexibility, which facilitates accelerated depolymerization and degradation rates (Inderthal et al., 2020). Thus, the expected differences in degradation rates for polymers with different degrees of branching can presumably be attributed to differences in crystallinity, i.e., differences in the degree of structural order in a solid substance. A material with disordered or random molecular arrangements

(lower crystallinity) occupies more volume and allows a higher flexibility of the molecular chains. The more crystalline the polymeric structure, more water and oxygen are needed for degradation. Therefore, an increase in the degree of crystallinity (related to increases in melting temperature and molecular weight of the polymer) reduces the degradation rate (Jenkins and Harrison, 2008).

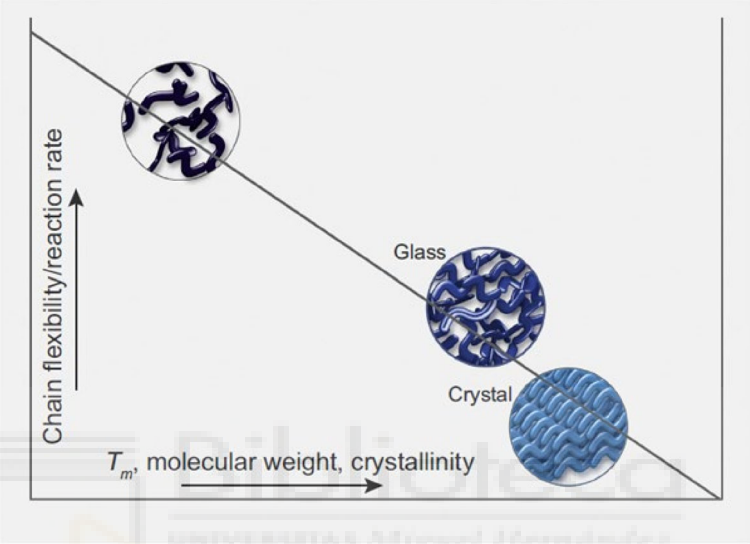


Figure 2. Flexibility-chain hypothesis (Inderthal et al. 2020).

On the other hand, the amorphous polymeric structure can be attacked by water and oxygen. The polymer amorphous regions are also considered more suitable for thermal oxidation (Li et al., 2019). As an example, extensive alkyl branching in low-density polyethylene (LDPE) prevents tight ordering, lowers the crystallinity, and should thus improve degradability compared with linear low-density polyethylene (LLDPE), which contains only short chain branches that allow denser packing and thus higher crystallinity (Inderthal et al., 2020). Moreover, the use of additives (e.g. stabilizers) in the plastic composition tend to decrease degradation rate and chromophores (carbonyl and hydroperoxide groups) (Aldas et al., 2018). The presence of chromophores leads to photo-chemically generated radicals that initiate the photo-degradation due to the presence of many available sites for photo-oxidation (Ali et al., 2021). Likewise, the presence of metal-metal bonds may enhance the photo-degradation process due to the homolytical bond cleavage upon irradiation (Daglen and Tyler, 2010). On the other hand, the morphological features of plastic also influence the degradation rate of plastic, since

it tends to increase with rough surfaces, which are more suitable for biofilm formation than smooth ones (Booth et al., 2017).

In the environment, abiotic and biotic processes often work in tandem, with abiotic degradation leading to smaller molecules that are subsequently mineralized by microbes. In natural conditions, the main mechanisms for plastic degradation are photo-degradation, thermo-oxidative degradation, hydrolytic degradation and biodegradation (Andrady, 2011). The environmental degradation process usually starts with photo-degradation, followed by hydrolysis and thermo-oxidation process. These processes lead to plastic wastes breakage into low molecular weight compounds, which can subsequently be metabolized by microbial activity (Andrady, 2011; Webb et al., 2013). Even for conventional biodegradable plastics, biodegradation is a poorly controlled and understood mechanism influenced by the crystallinity, crosslinking, molecular weight, additives, but also by the environment (moisture, temperature, pH, microbial activity…). While different models, such as BIOWIN, BESS, METABOL, etc. exist for the prediction of the biodegradation of organic compounds (chemicals), no model is available for the prediction of the biodegradation of polymers. Due to the underlying complexity and number of influencing variables, and even more when talking about novel conventionally non-biodegradable plastics degradation, it seems unrealistic to envisage a mechanistic model that would properly describe the actual behaviour of such plastic item in our target new end of life process conditions. In contrast, data driven approaches can cope with such challenges. The "Deterministic" controllable test parameters and properties (polymer type, biotic or abiotic treatments) and the less controllable environment characteristics (moisture, temperature, oxidizing agent, microbial activity, etc.) can produce a sort of guidelines about the critical mechanisms that contribute to plastic degradation. In this section, these two main approaches (abiotic and biotic degradation routes) have been described separately to ease of understanding the different processes, despite being interrelated in the environment.

Abiotic degradation routes of agroplastics

The abiotic degradation mechanisms for plastics can be classified as either (i) physical, referring to changes in the bulk structure, such as cracking, embrittlement, and flaking, or (ii) chemical, referring to changes at the molecular level such as bond cleavage or oxidation of long polymer chains to create new molecules, usually with significantly shorter chain lengths (Chamas et al., 2020).

In accordance to La Mantia et al. (2017) the degradation routes of single polymers should be essentially attributed to the formation of macromolecular radicals, due to the action of some external driving forces (temperature, mechanical stress, radiations, etc.) and to the subsequent reactions of such radicals with both the polymer macromolecules and oxygen. The so-formed unstable oxygenated species evolve towards the formation of stable macromolecules with oxygenated groups and gives rise to a dramatic change of the molecular structure (molecular weight, polydispersity, branching, etc); more in details, decrease of the molecular weight, possible presence of branching and, in a few cases, formation of cross-linked structures occur.

In the environment, the main abiotic mechanisms for plastic degradation are photo-degradation, hydrolysis and thermal degradation. The degradation of polymers is mainly due to photo-oxidation and thermooxidation reactions (Martínez-Romo et al., 2015; Wypych, 1995), causing the chain scission and cross-linking of polymer backbone, the formation of carbonyls (C=O) and vinyl (CH_2=CH) groups, and, finally, changes in the conformation and crystallinity of the polymer (Hoekstra et al., 1997; Khoylou & Hassanpour, 2005; Scott, 2000).

a) *Photo-degradation or photo-oxidation*

This mechanism is considered the most important abiotic degradation pathway in aerobic outdoor environments (Gijsman et al., 1999).Photo-oxidation causes the oxygenation of the plastic surface, which increases the polymer hydrophilicity and enhances the microbial biofilm formation on the polymer surface (Ali et al., 2021) (Figure 3).

The sensitivity of polymers to photo-degradation is related to the ability to absorb the harmful part of the tropospheric solar radiation, which includes the UV-B terrestrial radiation (~295–315 nm) and UV-A radiation (~315–400 nm) responsible for the direct photo-degradation (photo-lysis, initiated photo-oxidation). Visible part of sunlight (400–760 nm) accelerates polymeric degradation by heating. Infrared radiation (760– 2500 nm) accelerates thermal oxidation (Gugumus, 1990; Pospisil and Nespurek, 1997). Most plastics tend to absorb high-energy radiation in the ultraviolet portion of the spectrum, which activates their electrons to higher reactivity and causes oxidation, cleavage, and other degradation (Shah et al., 2008).

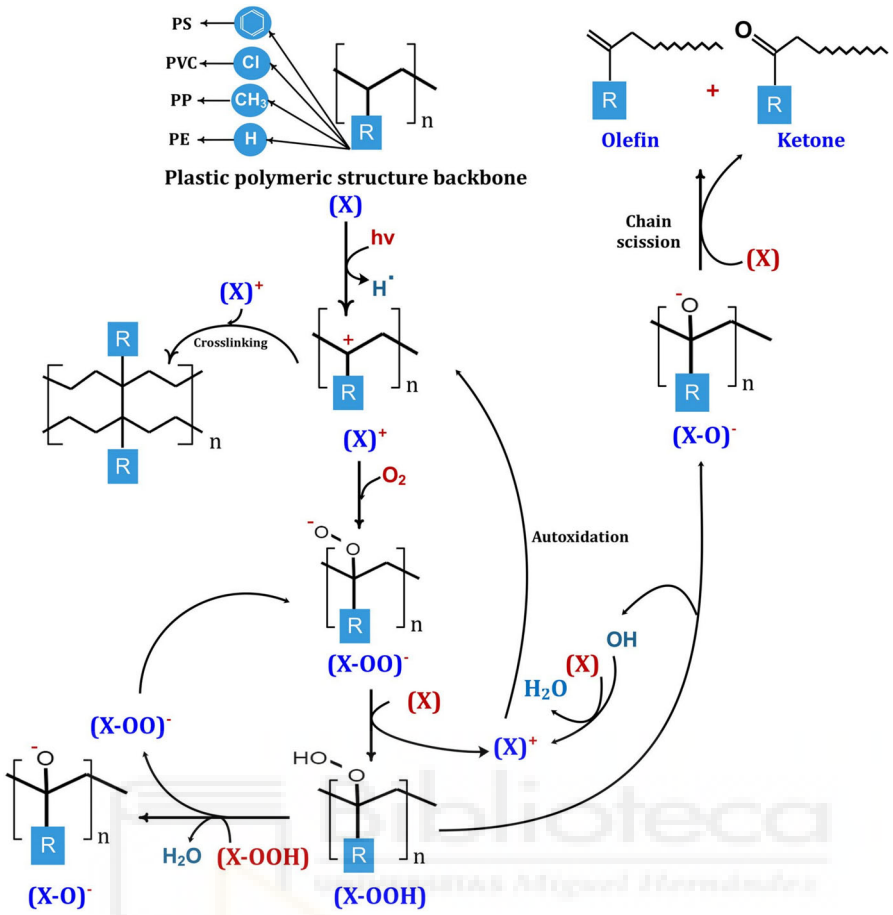


Figure 3. Plastic photo-degradation mechanism (Ali et al., 2021).

The mechanism of plastic photo-degradation includes three main phases: initiation, propagation and termination. During the initiation stage, polymer chain chemical bonds are broken by light or heat to produce free radicals (Yousif and Haddad, 2013). However, the polymers must contain unsaturated chromophoric groups that absorb light energy (Gijsman et al., 1999; Gewert et al., 2015). Although some plastics do not contain any unsaturated double bonds (e.g. PE and PP) and it would be expected to have a higher resistance to photo-degradation, the presence of small amounts of external impurities or structural abnormalities can allow the initiation of the photo-degradation process (Gijsman et al., 1999; Scott, 2002). Thereafter, in the propagation phase, the polymer radicals react with oxygen and form peroxy radicals. Apart from the formation of hydroperoxides, further complex radical reactions take place and lead to auto-oxidation (Singh and Sharma, 2008). Propagation ultimately leads to chain scission or crosslinking (Tolinski, 2009). Termination of the radical reaction occurs when inert products are formed from the combination of two radicals (Peacock, 2000). Thus, because of

oxidation, random chain scission leads to the production of oxygen-containing functional groups (e.g. olefins, ketones and aldehydes) (Scott, 2002). Since these compounds have unsaturated double bonds, are more susceptible to photo-initiated degradation.

b) Hydrolysis

Hydrolysis of plastic is considered one of the main steps during the abiotic degradation pathway (Ali et al., 2021). Hydrolysis is accelerated by the presence of catalysts, such as ions released through this reaction (Baes and Mesmer, 1976). The rate of this degradation mechanism depends on the susceptibility of polymeric chemical bonds to water attack and its concentration inside the material. Furthermore, the rate of water diffusion in the polymeric material is a critical factor (Crawford et al., 1988; Padsalgikar, 2017). During hydrolysis, water reacts with the polymer causing physicochemical changes and this process is chemically or biologically catalysed. In the acid-base catalyzed reactions), the mechanism involves a nucleophilic attack on the carbonyl group in esters or amides bonds (Hosseini et al., 2007). In addition, several factors seem to affect the hydrolysis rate, such as the molecular weight, decreasing the hydrolysis rate by increasing the molecular weight. Also, the reaction rate is affected by the molecules mobility and hydrophobicity or hydrophilicity (Booth et al., 2017). The combination of chemical and heat induces significant increase in degradability (thermochemical treatment).

c) Thermal degradation

Thermal degradation of polymers is ‘molecular deterioration as a result of overheating’ (Shah et al., 2008). The thermal degradation of plastic can be performed at high temperatures, usually higher than 100 °C, depending on the plastic polymer type and characteristics (Ali et al., 2021), such as the melting point and chemical structure. At high temperatures the components of the long chain backbone of the polymer can begin to separate (molecular scission) and react with one another to change the properties of the polymer (Shah et al., 2008). The combination of mechanical stress with thermal variation results in a “thermomechanical” degradation.

The chemical reactions involved in this abiotic mechanism produce physical and optical property changes relative to the initially specified properties (Shah et al., 2008). Thermal degradation generally involves changes to the molecular weight (and molecular weight

distribution) of the polymer and typical property changes include; reduced ductility and embrittlement, chalking, colour changes, cracking and general reduction in most other desirable physical properties (Olayan et al., 1996). On the other hand, the antioxidant additives incorporated through plastic manufacture prevents thermal oxidation at low temperatures. However, the degradation due to heat oxidation is accelerated by stress and exposure to other reactive compounds, like ozone (Ali et al., 2021). In general, the contribution of thermal degradation under normal environmental conditions globally is considered negligible, particularly in cold, marine environments (Kitamoto et al., 2011; Booth et al., 2017). In according to La Mantia et al., (2017) the main abiotic treatments to degrade plastics can be groped in five main types: Thermal/thermomechanical, photo-oxidation, chemical/thermochemical and other treatments.

a) Thermal/thermomechanical treatments

Thermal degradation can be defined as the process a polymer blend undergoes, in inert atmosphere, because of the action of heat. The effects can be very different depending on the components of the blends and, in turn, on their chemical structure. In general, thermal stresses result in the formation of decomposition (thermolysis) products which, depending on blend components, their relative amounts and the temperature, may initiate the degradation of the blend or, on the contrary, act as stabilizers (McNeill, 1989; Wypych, 1995). Synergistic (and thus, desirable) effects are present when the resistance to thermal degradation of the blend is higher than that of the individual components. In presence of oxygen (and thus, of air), thermo-oxidative degradation occurs; when also mechanical stress (typically, during processing) is involved, degradation is referred to as “thermomechanical”.

Thermal degradation efficiency depends on the melting point and chemical structure of plastic material/ingredients acting normally as a co-factor with oxygen and UV radiation in biodegradation processes in non-controlled environment. The number of branch points increases as polymer density decreases. Thermal degradation is normally monitored by using instrumental techniques based on thermogravimetry and others, measuring the loss of mass. TGA data has been popularly used in the past to study the thermal stability and kinetics of various polymers using different conditions. Weight loss determination and data analysis of experimental runs are crucial to determine the extent and type of governing mechanism of polymer decomposition (Al-Salem, 2018) (Figure 4):

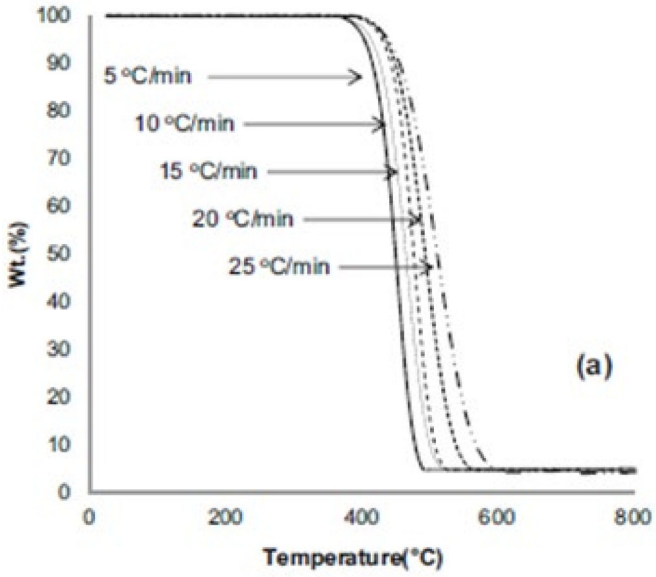


Figure 4. TGA curve from virgin LLDPE: loss of weight depending on temperature

Apparent activation energy is stablished depending on different models (CRIADO, Friedman, Coats & Redfern) to monitor thermal biodegradation, especially to stablish differences between conventional plastics vs. biodegradable or prodegradant chemical (e.g. with aromatic amines) derived plastics. Most of research studies are oriented to recycle or pyrolysis as a waste management strategy (La Mantia et al., 2017).

The thermodegradation of plastics involves both branching and crosslinking. Indeed, thermomechanical stress during multiple extrusions (recycling) did not cause relevant degradation phenomena; higher processing times in the mixer lead to significant increase of the viscosity and of the non-Newtonian behaviour, due to the formation of branching and cross-linked structures. For example, Wang et al. (2012) reported that the reprocessing of PP leads to a continuous reduction of the molecular weight, with the expectable effects on the melt flow index (increasing), the melting and crystallization temperatures (decreasing) and the crystallinity (increasing).

b) Photo-oxidation treatments

Photo-oxidation is only of the main pre-treatments to be used in plastic degradation, mainly due to the close relation to natural aging associated to the sun radiation, causing a general deterioration of their macroscopic properties due to the variation of molecular

weight, chemical structure, and morphology. Photo-oxidation treatments are based in the degrading action of radiation of the sunlight on the polymers. The energy of the radiation, correlated to specific wavelengths, results in the break of specific chemical bonds into de polymer structure. Then, depending on the energy, different types of chemical bonds can be broken, and act as starter for degradation cascades.

The principle of degradation states that the amount of energy absorbed by a molecule must exceed the bond energy in order to cause degradation. Photo-stability defines the degree of affection of plastic/blend to a specific radiation, normally sun radiation. UV-A radiation energy is not enough to break the chemical bonds of the PE and cause the degradation of the polymer (Wypych, 1995). UV-B radiation is particularly efficient in bringing about photo-damage in synthetic polymers. Although the UV-C has sufficient energy to break σ bonds, it is not a commonly used technique in the degradation of plastics because plastics materials lost their mechanical properties; furthermore, the UV-C radiation emitted by the sun is absorbed by the atmosphere and does not reach the earth's surface (Ojeda et al., 2009). Wypych (1995) reported the spectral sensibility of PE < 300 nm, and the activation wavelength at 300 nm.

In order to achieve an equilibrium between usability of plastics in environment and additionally facilitate the biodegradation of wasted plastics, increasing research and innovation have been done to include new formulations /additives / initiators to photo-oxidation processes. Figure 5 shows some of the combined processes to produce free radicals derived to the action of catalyst residue, light, heat, O_2 to obtain oxidized polymer. An overview of the interpretation of accelerated ageing behaviour under combined radiation-thermal oxidative environments has been presented by Celina et al (2019), concluding the complexity to stablish oxidation rates of plastics under radiative, thermal and aging combined stresses.

La Mantia et al. (2017) analysed the degradation behaviour of polymer blends depending on photo-oxidation, usually involving hydroperoxidation. They also concluded that the degradation rate of a polymer blend could result higher, intermediate, or lower than that of the pure components.

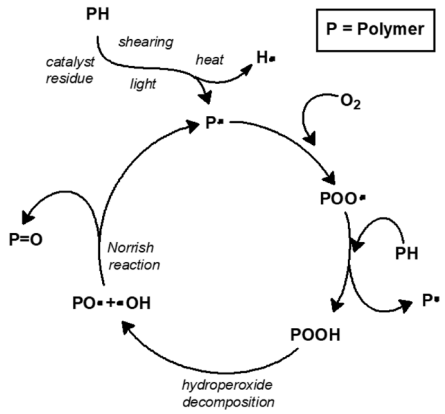


Figure 5. Free radical production derived of the action of catalyst residue, light, heat, O_2 to obtain oxidized polymer (Wypych, 1995).

c) Chemical/thermochemical treatments

Not that much significant research has been done on chemical degradation of AWP (De la Fuente et al., 2020), linked to environmental issues. In general, collection of plastic waste flows is mostly related to urban treatment plants, separate collected fractions and in these schemes, recycling of plastic wastes to produce new products (labelled as recycled in a certain percentage, vg. especially in PET plastics). However, when plastic wastes are a mixed blends and especially with specific origins vg. HDPE used for agricultural inputs, e.g., pesticides, fertilizers etc. the recycling option should be considered with care.

In addition, several industrial procedures should be done to prepare AWP for recycling or to reduce environmental hazards (presence of trace of pesticides, oils, other substances). Wienk et al. (1995) reported the use of hypochlorite as a nonspecific oxidizing agent observing chain scission for PVP membranes. De la Fuente et al (2020) reported that only Nitric acid and SDS treatment produced an increase in hydroxyl group peak in their experiment, showing the capacity of alteration-oxidation of LDPE (Figure 6). Rodriguez et al. (2021) using combined conditions of time, temperature and oxidizing chemical reagents obtained significant oxidizing effects including disintegration for the longest and highest concentration assays.

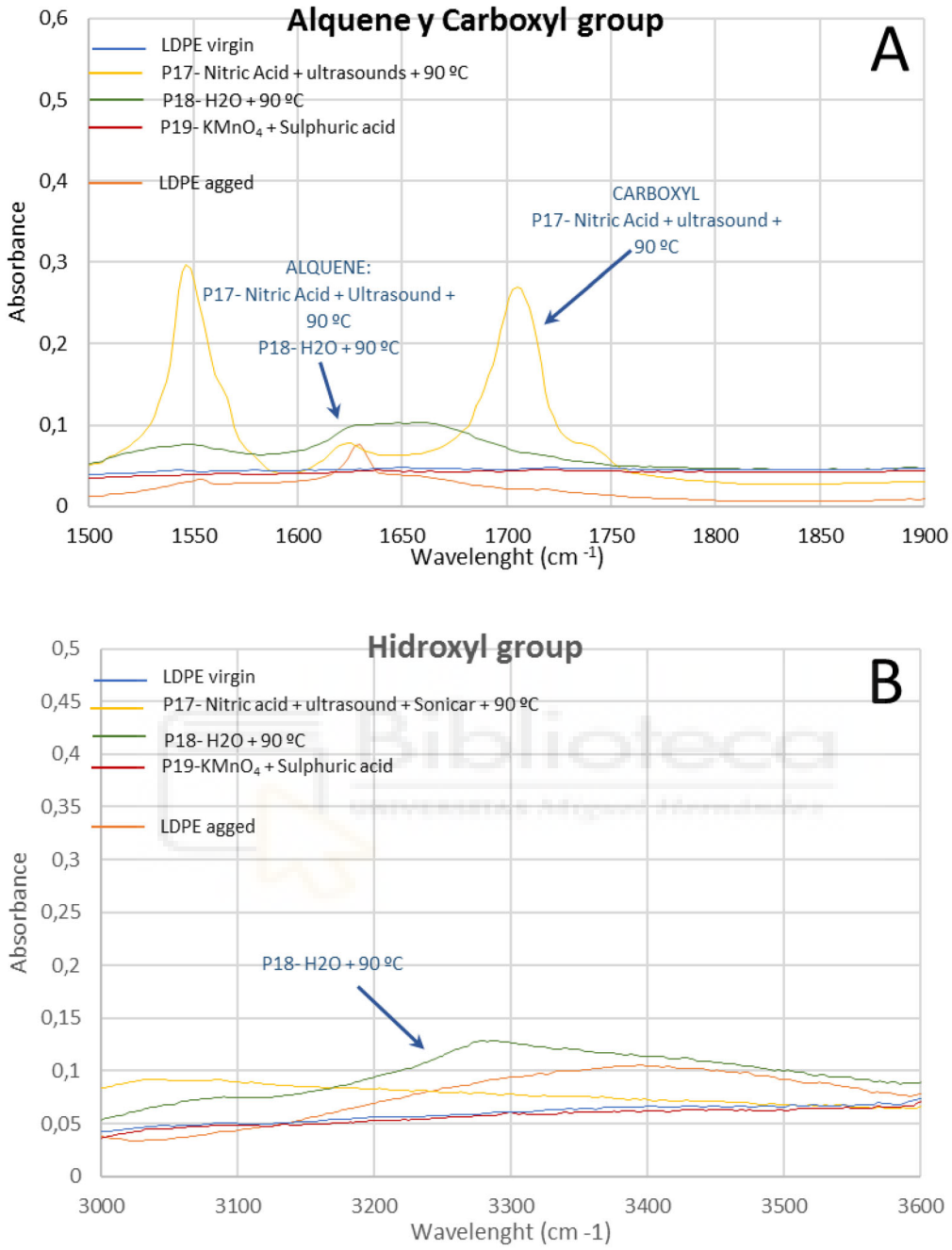


Figure 6. FTIR spectra for pre-treated LDPE in alkene and carboxyl region (A) and hydroxyl region (B) (de la Fuente et al., 2020).

d) Other treatments

Many other approaches, mostly related to new advanced instrumental procedures (electropulses, e-beam irradiation, supercritical conditions, etc.) normally used in other fields (food technology, pathogen removal) has been studied for new uses and specially

its effect on plastics as food packaging materials. In our thesis, we will study the e-beam technology for pre-treatments of plastics.

The history of the electron beam (e-beam) began in the 1870s with the experiments conducted by Crookes and Hittorf. In their first experiments, they tried to melt metals using an electron beam. Later on, more experiments have been carried out in which electron beam had been used for soldering, melting and welding. This led to the first electron beam processing machine built by Dr. h.c. Karl-Heinz Steigerwald in 1952 (Anonymus, no date).

The examination and modification of materials have developed continuously over the past decades. Electrons are negatively charged and very light particles. When subjected to an electric field, the kinetic energy gained from their acceleration can be converted into thermal energy. The amount of energy has to be adapted on the desired processes, as melting, cutting, or heating require different amounts of energy. Applications of e-beam technology are diverse. It serves in many fields, *inter alia*, lithography, manufacturing, medical, communication, entertainment, and sterilization. (Negi et al., 2019)

Although different kinds of e-beam systems are available, they have several parts in common. Those are electron beam guns, vacuum systems, and focusing and deflection electrodes. This set-up is placed into one vacuum chamber. The process begins by using an accelerator to create a beam. Generated electrons are extracted from an electron gun in a vacuum system. In the next step, the electrons are accelerated and scanned to a beam through a magnetic field. The penetration of the electron beam into the product depends on the voltage of a magnetic field. The electrons then exit the vacuum system through a specific foil and go to the product transported through the beam of electrons. (Anonymus, 2014).

Similar to other radiation technologies, the use of e-beam made it possible to modify and potentially improve various properties of polymers. During e-beam processing, either the molecular weight in-creases (due to cross-linking) or the molecular weight decreases (due to chain scission), which results in the development of the shorter molecule with higher mobility. As mentioned, the penetration depth of e-beam depends on the voltage and is therefore limited, and the absorbed energy decreases with increases depth. Therefore, the thickness of the material should be appropriate for the e-beam penetration level, regardless of the state of irradiated material (granules, film forming solutions, dry

films). The result of irradiation depends not only on the molecular organization but also on the presence of O_2, which promotes the formation of free radicals. (Zivanovic, 2015)

As summarized by Dawes et al. (2007), based on the molecular structure, some polymers are more prone to cross-linking and others are more prone to chain scission. The degree of unsaturation in the polymer chain, for instance, can enhance the effects and increase the yields of crosslinking as in the case of purified natural rubber (cis-1,4-polyisoprene). Whereas in the case of poly-isoprene composed of different isomers undergo a high yield for loss of unsaturation upon irradiation. On the other hand, the greater the amount of aromaticity present in the polymer chain, the lower the yield of any reaction that occurs as a result of irradiation. Therefore, polystyrene only shows very little yield of both chain scission and cross-linking. Polyethylene on the other hand is more prone to crosslinking than chain scission. (Dawes et al., 2007) However, this also differs for LDPE and HDPE, for example (Vasile and Butnaru, 2017). For polyesters, the dominant effect of irradiation is chain scission (Dawes et al., 2007).

Radiation can also cause the formation of radiolysis products, where the products and their concentrations again depend on the polymer. In the case of PE, peroxy radicals form on the surface. This leads to an oxidative decomposition of the polymer and of additives, which in turn can result in an increase in low volatile compounds. Radiolysis products may also be trapped in the polymeric structure. If peroxy radicals continue to react, this can promote chain scission and the generation of further radiolysis products. Notably, in solid state, these radicals can be found for months after radiation treatment. Therefore, protective additives may be used for commercial polymers. However, plasticizers can have a similar protective effect. (Vasile and Butnaru, 2017)

Upon irradiation in air, oxidized physicochemical groups and the molecular modifications occur in solid polymer. The extent of these modifications and of oxidative reactions depend on the reaction kinetics, which in turn depend on the oxygen diffusion rate through the polymer. A more uniform oxidation within the material occurs when the molecular structures, morphologies and irradiation conditions favour the oxygen diffusion in the polymer. When oxygen diffusion is hindered, these alterations do not occur uniformly within the material. As the absorbed dose as well as the absorbed energy decrease with increasing material depth, external layers then exhibit a higher extent of oxidative degradation. Moreover, similarly to molecular weight modifications, the formation of a gradient reflecting the concentration of oxidized groups can be observed. (Spadaro et al., 2017)

There are several possible processing techniques of polymers using e-beam technology, such as curing, cross-linking, scissoring, and grafting. Peculiar to the curing process is a rapid solidification of ink, liquid coating, or adhesive. As mentioned above, during an e-beam process, radicals are formed, which on the one hand are able to initiate the polymerisation of monomers and the formation of polymer networks in the cured state. On the other hand, depending on the polymer, the radiation settings and atmosphere, chain scission occurs, if these radicals fail to recombine and are terminated by reactions with oxygen and/or hydrogen abstraction. (Lapin, 2015) Therefore, e-beam processing was also investigated as tool to control degradation profiles of polylactide acid films. This study showed that the higher the radiation energy in terms of voltage and the higher the radiation dose, the more the degradation accelerated. (Cairns et al., 2011) These findings demonstrate the potential of e-beam treatment as acceleration tool for polymer degradation.

Biotic degradation routes of agroplastics

The biodegradation of plastics in the environment is a complex process that involves a combination of many abiotic and biotic factors. After fragmentation or modification accomplished by abiotic factors and microorganisms, the bulk polymer becomes more available for biological attack. Extracellular depolymerizing enzymes from microorganisms, release monomers that are further intracellularly metabolized by them. Consequently, microorganisms and their enzymes are ultimately responsible for the complete mineralization, which is the transformation to carbon dioxide (CO_2), and the final removal. The biotic degradation then must be initialized by ageing or processes that reduce crystallinity and packaging of polymers (1-2) to obtain hydrolysable plastics (able to be degraded under the action of exocelullar enzymes, and therefore the degradation products (monomers) be absorbed into the microorganism cell to be used as metabolic substrate and finally plastic derived chemical elements, used into the microorganism tissue or emitted as CO_2 or H_20 (Figure 7).

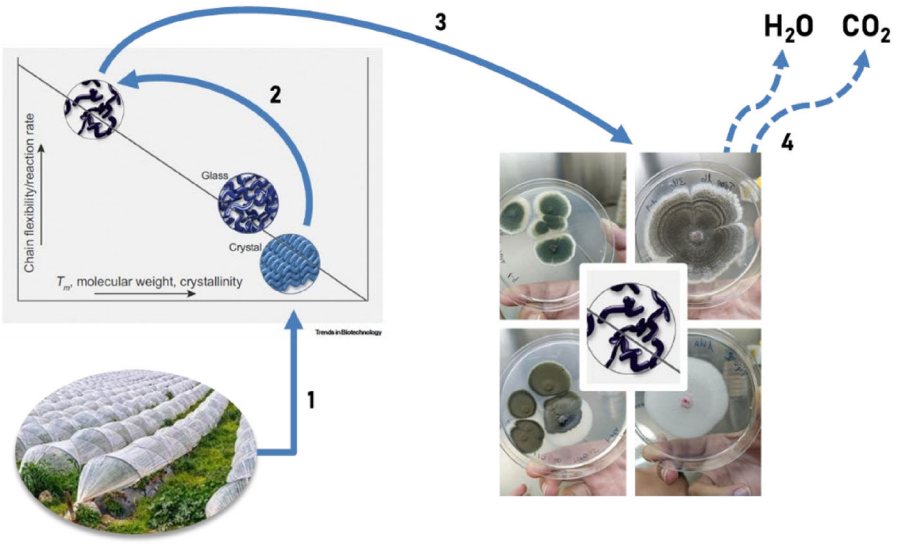


Figure 7. Scheme of biodegradation of plastics.

The main limitations for the biodegradation of plastics include the high molecular weight of polymers, the lack of favourable functional groups, and the high degree of crystallization. Therefore, long-chain polymers cannot cross the cell membrane and must be treated with extracellular enzymes (Wilkes and Aristilde, 2017). Synthetic plastics tend to be highly hydrophobic as they present stable functional groups such as alkane and phenyl. Consequently, oxidation and hydrolysis are necessary steps to increase hydrophilicity and promote microbial attack (Shah et al., 2008). Likewise, plasticizers and additives significantly influence the biodegradability of polymers. Therefore, the degradation of microplastics consists of an event composed of various physical-chemical and microbial factors that occur naturally in the environment (Urbanek et al., 2018). In this way, microplastics can act as constituents of an ecological niche that allows the colonization and growth of organisms, offering an important source of carbon to use (Yuan et al., 2020). However, this capacity is only available for those organisms that have enzymes capable of incorporating the products obtained, monomers of plastic polymers, in the corresponding metabolic routes for obtaining energy (Pham et al., 2021).

Research on the biodegradation of recalcitrant synthetic polymers with carbon-carbon backbones, namely PS, PVC, PP and PEs, has proved that they can be attacked, under different lab and actual site conditions, by a variety of mixed microbial communities and enzymes and pure fungal or bacterial strains obtained from terrestrial or aquatic environment and from some earthworm and insect's larval gut. Earthworms and insect larvae are also able to degrade plastics in which process, their gut microbiota play a key

role. Most work in this area has been focused on PUR and PET that are easier to biodegrade but biodegradation of other more recalcitrant plastics or mixtures are also scarcely covered (Raddadi and Fava, 2019).

A wide bacterial biodiversity has been reported to degrade plastics (Matjašič et al., 2021). Among all of them, one that stands out as a potential candidate for plastic biodegradation is Pseudomonas because of its diverse metabolic and stress-resistance capabilities and genetic plasticity (Wilkes and Aristilde, 2017). Together with the Bacillus isolates, these are the most active strains against different plastic structures. The biodegradation of plastics is typically a surface erosion process due to difficulty in penetration of extracellular enzymes into the polymer and so act only on the polymer surface (Kale et al., 2015). The most common modifications of plastic polymers by bacteria are weight loss, changes in the tensile strength and chemical and surfaces changes. Several fungi also have the potential to degrade plastics in aquatic and soil environments (Brunner et al., 2018). Aspergillus, Fusarium, Pycnoporus and Mucor are among those proposed for the biodegradation of plastics. In addition, fungi capable of degrading lignin in nature are of particular interest with respect to particularly inert structural elements of plastics such as C–C or C–Cl bonds. Lignin resembles certain plastics in not being hydrolysable and possessing similar structural elements that are oxidized and cleaved during lignin degradation by enzymes like laccase and lignin peroxidases that might enable them to degrade the C–C bond-based PE and PP (Krueger, 2015). Laccase and MnP enzymes secreted by fungus *Chaetomium globosum* were responsible for degradation of polyethylene (Sowmya et al., 2014).

To date, the rates achieved by pure microbial cultures have been low and almost all isolates are restricted to growth on a single type of plastic under optimal lab scale conditions (Paco et al., 2019) (Table 2). Consequently, microorganisms and enzymes are needed that can more rapidly and efficiently degrade complex plastic mixtures.

Microbial consortia can improve degradation capabilities and promote the degradation of a more diverse group of plastics (Drzyzga and Prieto, 2019). For example, the decomposition of micro-sized polyethylene (PE) by mesophilic mixed bacterial culture obtained from a municipal landfill sediment reduced the dry weight of particles (18% after 60 days) (Park and Kim, 2019). Also, microbial communities isolated from plastic garbage processing areas has been used for enhanced degradation of low-density polyethylene (LDPE) strips and pellets with weight reductions of 81% and 38%, respectively, after 120 days (Skariyachan et al., 2017).

Table 2. Maximum biodegradation of synthetic plastics reported in literature for microbial pure culture (Coltelli and Aglietto, 2015).

Plastic	Microorganisms	Weight loss (%)	Period
PUR	*Comamonas acidovorans*	48-100	7 days
PE/LDPE/HDPE	*Zalerium maritimum*	70	21 days
PET	*Ideonella sakiensis*	100	6 weeks
PP	*Pseudomonas stutzeri*	0.25	60 days
PVC	*Pleurotus* sp., *Phanerochaete chrysosporium*, *Poliporus versicolor*	0.55	30 days
PS	*Bacillus* sp.	23	30 days
PC	*Phanerochaete chrysosporium*	5.4	12 month

The use of microbial enzymes for the biodegradation of plastics is another highly valuable option. The diversity of known enzymes and microbes acting on synthetic polymers is still rather limited (Danso et al., 2019). The enzymes reported to be involved in the breakage of polymers are cutinases, esterases, laccases, lipases and enzymes involved in lignin metabolism (Inderthal et al., 2020). Despite the lack of useful enzymes compared to high-density plastics (PS and PVC), there are enzymes with moderate degradative activity for low-density plastics. Using a combination of enzymes is also known to enhance polymer hydrolysis (Bhardwaj et al., 2013). Furthermore, it is possible to generate hydrolytic enzymes in large quantity at low cost (Dutta et al., 2009). In addition, the design of novel enzymatic degradation modules employing plastic hydrolases with high rates of plastic depolymerization is possible.

According to these studies, it is feasible to induce a specialized plastic-degrading microbial community in the gut of mealworms and to improve their capabilities by adjusting rearing conditions. Finally, the possibility of modifying and increasing capabilities of the microbiome within these organisms by feeding them with microorganisms is a subject to consider. Recently it has been shown that inoculating insects with symbiotic bacteria *Enterobacter cloacae*, *Enterococcus faecalis* provide a readily transferable tool for insect rearing systems that improve fitness by providing additional protection against infection, or by normalizing insect physiology increasing survival rates (Somerville et al., 2019).

1.4. Organic waste management and circular economy

Circular economy appears in the literature mainly through three fundamental actions known as the 3R principles: Reduce, Reuse, Recycle (Ghisellini et al., 2016), becoming a powerful integrated climate change mitigation strategy. The utilization of raw materials that are currently disposed of as waste is one of the basic principles of the circular economy and also one of the main objectives of the European Union's 2030 agenda for sustainable development. The aim is to manage waste with greater prevention, reuse and recycling in order to open up new opportunities for reducing greenhouse gas emissions and mitigating climate change. Other objectives for establishing a circular economy lie in profound changes to the current production and consumption model.

On 2 December 2015, the European Union adopted for the first time a package of legislative proposals to boost the transition to the circular economy "Closing the loop: an EU action plan for the circular economy" (EC, 2015). This action plan sets medium- to long-term objectives for organic waste, such as non-landfill and incineration of the vegetable biomass produced. The Communication notes in paragraph 6 that support for research and innovation will be an important factor in encouraging the transition to a circular economy, and will contribute to the competitiveness and modernisation of EU industry.

To achieve these objectives, it is necessary to recover bio-waste (a pillar of the Circular Economy), such as agro-industrial waste (from waste to resource) and thereby increase the life cycles of agricultural biomass, extracting the maximum value from it, as well as defining a better waste hierarchy. Importantly, the waste hierarchy also broadly reflects the environmentally preferable option from a climate point of view: disposal, either in landfill or by incineration with little or no energy recovery, is typically the least favourable option for reducing greenhouse gas (GHG) emissions; in contrast, waste prevention, reuse and recycling are the processes with the greatest potential to reduce GHG emissions.

The “Proposal for a REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL” establishing provisions relating to the marketing of fertilizing products with the CE marking, amends Regulations (EC) No. 1069/2009 and (EC) No. º 1107/2009” (EC, 2016) and Regulation (EC) No. 2003/2003 is repealed, for its subsequent publication as a new “EU Regulation on fertilizers, limestone amendments, amendments, growing medium and biostimulants” 2019/ 1009. This new Regulation allows the free circulation of fertilizers throughout the European territory, promoting circular agriculture

and sustainable agriculture, as well as promoting greater use of recycled materials and reducing waste and dependence on imported nutrients. This new regulatory framework that aims to establish a unique scenario that allows the production of fertilizers from recycled bio-waste and other recycled raw materials, as reflected in point 19 of the communication, provides innovative solutions for a more efficient and safe valorization of the resources coming from of waste, water and bio-waste.

There are different types of organic solid waste (OSW) treatment, depending on the type of recovery carried out. In its transformation, a new raw material can be obtained, used as biofertilizer (which will be incorporated back into the production process, completing the cycle of the circular economy), or, on the other hand, energy recovery is carried out, obtaining energy from its recycling, such as biogas or biomethane. The following typologies can be mentioned: incineration, pyrolysis, biomethanization, anaerobic digestion, controlled landfill, anaerobic digestion, composting, vermicomposting, and bio-drying (Table 3).

Table 3. Types of organic waste valorization.

Types of OSW	Treatment	Process	Output	Advantage	Disadvantage
Waste disposal	Controlled landfilling	Burial of waste in designed areas. Landfills can be either underground or above-ground	Waste disposal system	Simple management	Generates bad Odors. It is the least circular management method.
Energy recovery	Incineration	Waste is incinerating, i.e., thermally destroying it to the point where it is transformed into combustion gases or products such as ash	Energy & ashes	In specialised plants, its possible to obtain large amounts of energy	Ash generation
	Pyrolysis	Waste is thermally treated in a sealed container under high pressure. Thermal degradation is done with a very limited amount of oxygen	Gases, liquids, and inert materials	This system is greater than that of incineration, since pyrolysis produces much greater amounts of energy	Treatment of high economic cost
Biofertilizer	Composting	Aerobic biological process that, under controlled conditions, transforms degradable organic waste into a stable and sanitised material called compost	Compost, GEI & humidity	Compost can be used as an organic amendment. This treatment favours the return of organic matter to the soil and its reintegration into natural cycle.	Need to optimize the mix of waste to be composted
	Vermicomposting	Degradation of organic waste by earthworms	Compost, GEI & humidity	Vermicompost can be used as an organic amendment	Maintain optimal conditions for living organisms
Biofertilizer & energy recovery	Biomethanization	Anaerobic digestion of organic fraction of waste	Biogas	Can be used as a renewable gas for residential, industrial or mobility uses	Maintain optimal conditions for living anaerobic organisms
	Bio-drying	Consists of evaporating part of the moisture contained in the organic waste and stabilising it	Organic matter partial degraded, evaporation of the water contained, and pathogens are eliminated	This system can be used with mixed fractions without prior mechanical sorting treatment. The reduction of moisture content <20% by weight generates a high PCI material that could be valorized as fuel	Treatment with slightly high economic cost
	Anaerobic digestion	Process by which microorganisms decompose biodegradable material in the absence of oxygen	Various gases (CO_2, CH_4 etc.) & digestate	The gases are used as fuel and the digestate as organic soil amendment	Complex process and economically costly installations

The composting/vermicomposting of agricultural waste has been widely studied and in recent years, it is being optimized and formed as a suitable agro-industrial solid organic waste recovery system. On the one hand, the growing demand for soilless crop production and the associated problems of using peat, have led to the need to look for alternative materials for the agricultural sector and low-cost substrates as a horticultural growing medium, such as the use of compost as a substrate (Abad et al., 2001). On the other hand, its use as an agronomic application has also expanded. This organic amendment has a multitude of beneficial effects on the soil, such as restoring C reserves in soils, maintaining higher levels of soil organic matter (especially important in arid and semi-arid regions), providing nutrients and enhances microbial proliferation and activity (Tejada et al., 2006). The capacity as a C sink of soil organic matter for atmospheric CO_2 represents a brake on the greenhouse effect, with a C sequestration potential in arid zones of approximately 0.10 – 0.20 tons C. / ha / year for application rates of 20 mg / ha / year (Lal, 2004).

It should be noted that for the treatment of organic solid waste to be effective, it is important to properly separate the waste generated at the source and deposit it in the containers provided for each type of waste. The increasing use of agroplastics, their inadequate management and pollution from diffuse sources (facts that are discussed in depth in previous sections), are responsible for organic waste being contaminated by a large amount of plastics or microplastics. This fact makes its management and valorization difficult, such as through techniques such as composting or vermicomposting (Figure 8).

Figure 8. Composting and vermicomposting process.

1.5. Earthworms and ecological categories

Earthworms are important components of the soil system, mainly because of their favourable effects on soil structure and function (Paoletti 1999; Jongmans et al. 2003). It belongs to the phylum Annelida, order oligochaeta (Edwards and Lofty, 1977). Currently, there are more than 7,245 species of Oligochaetes that have been classified worldwide (Reynolds, 1998). Moreover, these organisms comprise the majority of invertebrate biomass (> 80%) in terrestrial environments and have over 600 million years of evolutionary experience as “environmental engineers” (Fierer, 2019). Their burrowing and feeding activities contribute notably to increased water infiltration, soil aeration, and the stabilisation of soil aggregates. Earthworms also provide multiple environmental services in agrosystems.

Earthworms can be classified into three ecological categories, depending on the ecosystem functions and services they perform. They can also be grouped into two large groups: earthworms that mainly live on the surface within litter and/or wet organic matter (epigeic species) and earthworms that feed mainly on the mineral fraction of the soil (anecic and endogeic species). This classification, used worldwide, was described for the first time by Marcel Bouché at the beginning of the 1970s. The concept was first introduced in a chapter of an edited book (Bouché, 1971), further explained in detail in his book (Bouché, 1972), and finally summarised again in a conference paper a few years later (Bouché, 1977). This last paper was particularly successful since it described, for the first time, a triangle defined by three poles corresponding to three main ecological categories and positioned some typical earthworm species within this triangle. Other authors have introduced new intermediate categories; for example*, Lumbricus terrestris*, which is cited primarily as anecic species, is sometimes classified as epi-anecic.

Epigeic species live in the first 30 cm of the soil and feed on organic matter, producing humus on the surface and forming macropores. On the other hand, anecic species live in vertical burrows, and endogeic species live in horizontal burrows in mineral soil. Epigeic earthworms are often used for the decomposition of organic waste in vermicomposting, as will be described in more detail in the next section. Table 4 shows additional information on the main characteristics of each category.

Table 4. Characteristics of ecological categories of earthworms

Characteristics	Epigeic species	Anecic species	Endogeic species
Habitat	3-10 cm, surface dwellers	30–90 cm, deep burrowing	10–30 cm, upper layer soil
Body size	Small	Large	Moderate
Colour	Uniform body coloration	Pigmentation only at the anterior and posterior end	Weak pigmentation
Life cycle	Short	Long	Medium
Temperature tolerance	Tolerant a wide range of temperature	Poor temperature tolerance	Poor temperature tolerance
Live in	Near the surface litter or dung	Deep soil	Below the surface
Reproduction rate	High	Moderate	Low
Feeding habitat	Plant litter or mammalian dung. Undecomposed litter	Decomposed litter, surface litter	Organic rich soil, subsurface soil material
Major role	Efficient bio-degraders and are good for vermicomposting	Distribution and decomposition of organic matter in soil	Soil mixing and aeration processes
Vermicomposting potential	Good	Low	Low
Common species	*Eisenia fetida, E. andrei, Eudrilus eugenie, Lumbricus rubellus, L. festivus, L. castaneus, Bimastus eiseni, B. minusculus, Drawida modesta, Dendrodrilus rubidus, Dendrobaena veneta and Perionyx excavatu*	*Lumbricus terrestris, L. polyphemus, Lampito mauritii, Apporrectodea trapezoids and A. longac*	*Octochaetona thurstoni, Aporrectodea caliginosa, Allolobophora rosea, A. caliginosa, Metaphire posthuma, Pontoscolex corethrurus, Drawida barwelli and Amynthas sp.*

Epigeic earthworms live in the organic horizon, or near the soil surface, and feed primarily on decaying organic matter, such as plant debris (litter), decaying plant roots, or dung. They cause a limited mixture of mineral and organic layers when feeding on bedding materials. They differ from the rest mainly by not making burrows and living on the surface of the organic profile of the soil. They tend to be small (1-18 cm in length) and have high metabolic and reproductive rates that allow them to adapt to the changing environmental conditions of the soil surface. They also display high rates of consumption, digestion, and assimilation of organic matter and play a key role as litter transformers, producing holorganic casts. These species are usually bright red or reddish brown but are not striped. The pigmentation acts as camouflage as they move through the leaf litter. It also helps to protect them from UV rays. Being close to the ground surface makes them vulnerable to stock treading in intensively grazed paddocks, as well as exposing the earthworms to predators; however, their muscles are strong and thick in proportion to their length, allowing for quick movement. This ecological category includes the following species: *Allolobophoridella eiseni, Bimastus eiseni, Bimastus minusculus,*

Dendrobaena attemsi, Dendrobaena hortensis, Dendrobaena octaedra, Dendrobaena veneta, Dendrodrilus rubidus, Drawida modesta, Eisenia andrei, Eisenia fetida, Eiseniella tetraedra, Eudrilus eugenie, Helodrilus oculatus, Lumbricus castaneus, Lumbricus festivus, Lumbricus friend, Lumbricus rubellus, Metaphire californica, Perionyx excavatu and *Satchellius mammalis*.

Introduced epigeic earthworms tend to live in compost (such as the introduced tiger earthworm *Eisenia fetida*, which cannot survive in soil) and under logs and dung. Native species usually live in forest litter. In agricultural soils, earthworms usually burrow deeper than they do in grasslands and forest soils. Temporarily very high densities can be found in forests (> 3200 ind. m^{-2} reported by Dymond et al. 1997) due to changes in seasonal soil conditions (such as variation in humidity, temperature, etc.). In nature, epigeic species occupy unpredictable and unstable habitats, characterised by highly variable environmental conditions, food availability, and predation pressures. When conditions are unfavourable, epigeic earthworms suffer high mortality, the population density oscillates widely, and the reproduction rate increases greatly (Monroy et al., 2006). Under these circumstances, the ability to grow and reproduce exponentially is critical.

From the point of view of their life history, epigeic earthworms are typical “r-strategists” or fast developers in the slow-fast continuum. Fast or r-selected organisms have typically short life cycles, are small, attain sexual maturity rapidly, and have high metabolic rates. Under unfavourable environmental conditions, high reproduction rates will ensure population survival, and the formation of cocoons may enable the earthworms to resist until conditions become more favourable, thus explaining the fluctuations in population density.

The short life cycles, the ability to reach sexual maturity quickly, their high metabolic rates, and their continuous exposure to the environmental conditions of the soil are some of the characteristics that make these species used as model species in laboratory tests. Two of the most used species in this ecological category correspond to *Eisenia fetida* and *Eisenia andrei*. They are closely related earthworm species that are widely used in vermicomposting systems to recycle organic waste, as well as in ecotoxicological, physiological, and genetic studies. These species are widely used because they are ubiquitous, have short life cycles, high reproduction rates, are tolerant to a wide range of temperature and humidity, and are relatively easy to handle (Dominguez, 2004; Dominguez and Edwards, 2011).

Both species were originally described as different morphotypes of *E. fetida* according to differences in body pigmentation. Bouché (1972) later gave these earthworms subspecific status, naming them *E. fetida fetida* and *E. fetida unicolor* (Bouché, 1972). Although many

authors now accept that *E. fetida* and *E. andrei* are different species, the oldest literature and much current literature refer to these species collectively as *E. fetida* or *E. fetida*, an incorrect version of the original *E. fetida* (Sims, 1983; Easton, 1983). *Eisenia fetida* is the striped morph, and the area between the segments has no pigmentation or is yellow or pale yellow, hence its common name of striped worm or tiger worm. By contrast, *E. andrei*, the common red worm, is uniformly red in colour. Apart from the differences in pigmentation, the species are morphologically similar (Domínguez, 2018) with no differences in biological parameters, especially in relation to reproductive potential and life cycles, although the rates of growth and cocoon production are somewhat higher in *E. andrei* than in *E. fetida* (Elvira et al., 1996). The external morphological characters (Figure 9) are commonly used to distinguish between earthworm species (Dominguez, 2018).

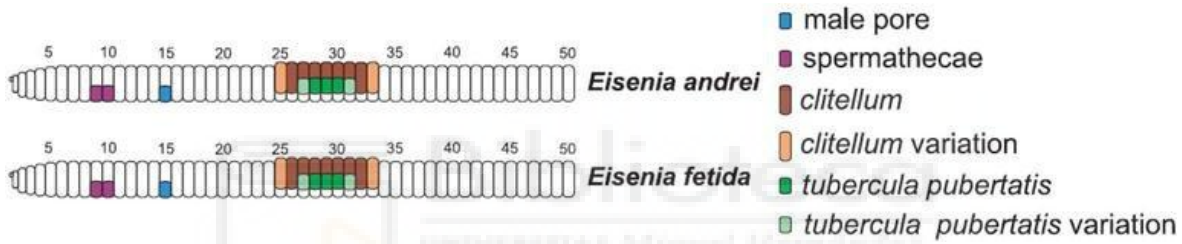


Figure 9. Diagram of the external morphology of *Eisenia andrei* and *Eisenia fetida*

The optimum temperature for growth of both species is 25°C, and although they can tolerate a wide range of moisture conditions, the optimum moisture content for these species is 85%. In optimum conditions, the length of their life cycles (from newly laid cocoon through clitellate adult earthworm) ranges from 45 to 51 days. The time for hatchlings to reach sexual maturity varies from 21 to 30 days. Copulation in these species, which takes place beneath the soil or waste surface, has been mentioned by various authors since 1845 and has been observed more often than in any other megadrile species. Cocoon laying starts 48 hours after copulation, and the rate of cocoon production is 0.35 – 0.5 day^{-1}. The hatching viability is 72% – 82%, and the incubation period ranges from 18 to 26 days. The number of young earthworms hatching from viable cocoons varies from 2.5 to 3.8 depending on the temperature. In controlled conditions, the average life span is 594 days at 18°C and 589 days at 28°C with a maximum life expectancy between 4.5 and 5 years, although under natural conditions it may be considerably shorter.

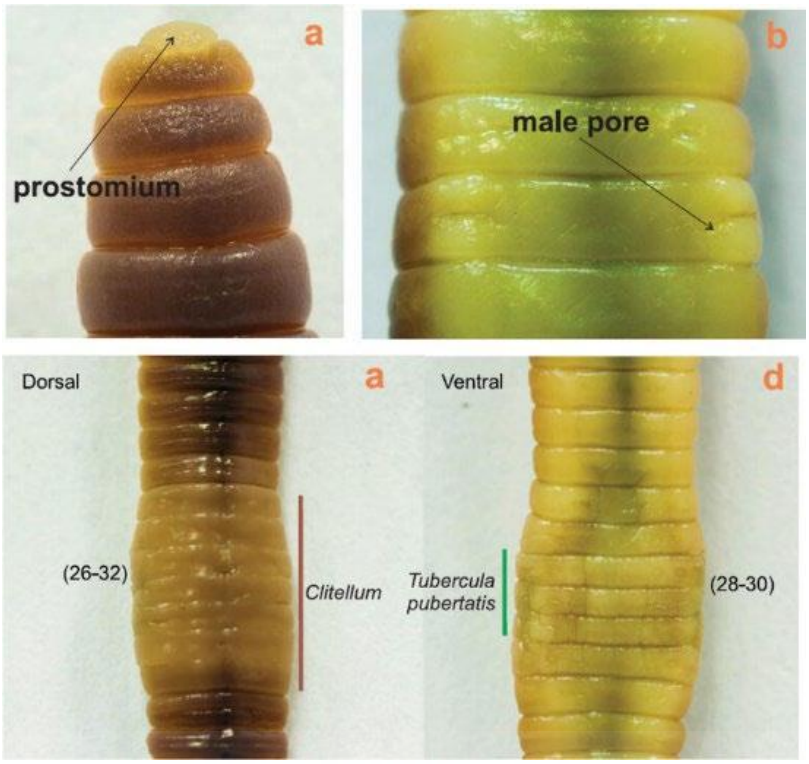


Figure 10. External morphology of *Eisenia andrei*. (a) Dorsal view of prostomium, peristomium, and first segments. (b) Male pores in the ventral side of segment 15. (c) Dorsal view of the clitellum in segments 26–32. (d) Ventral view of the tubercula pubertatis in segments 28–30.

Although they are very similar, *E. andrei* and *E. fetida* are biologically different species, and consequently, the coexistence of both species in mixed cultures inevitably leads to poorer functioning of the vermicomposting system. The abundance and frequency of citations in specialised and nonspecialised literature that indiscriminately refer to *E. andrei* and *E. fetida* as different names for the same species suggest that mixed cultures of both species are also quite common. In mixed cultures, the reproduction rate and biological efficiency will be much lower than in pure cultures because earthworms will waste energy carrying out unsuccessful copulations.

Other species that can be used in vermicomposting are mentioned below: *Eudrilus eugeniae* (Kinberg, 1867) is a large earthworm, native to Africa, very fast-growing and reasonably prolific (Viljoen and Reinecke, 1989). Under optimal conditions, it would be an ideal species for protein production. Its main disadvantages are its sensitivity to low temperatures (Reinecke et al., 1992) and its weakness, which makes it difficult to manage. *Perionyx excavatus* (Perrier, 1827), is a highly prolific species, easy to manage, suitable for protein production (Guerrero, 1983), and efficient in the degradation of waste (Kale et al., 1982). Its main disadvantage is its

poor tolerance to low temperatures (Reinecke et al., 1992); *Dendrobaena veneta* (Rosa, 1886), although a large species, seems less suitable than others due to its low reproductive rate (Loehr et al., 1985) and rather slow growth. It is demanding in terms of environmental conditions, tolerating only moderate temperatures (Edwards and Bater, 1992) and a humidity range between 65 and 80%, with high mortality rates outside these indices (Muyima et al., 1994); *Lumbricus rubellus* (Hoffmeister, 1843), which presents similar problems to *D. veneta*, is not sufficiently well studied. It could be of interest from the point of view of protein production and live bait for fishing.

The characteristics and life history aspects of eight common epigeic species of earthworms are summarised in Table 5. We decided to preselect *Eisenia fetida* for the following reasons: being used in many experiments as an ecotoxicological model, having high reproduction, being easy to incubate in the laboratory, having high viability, having a medium life cycle, and it's capable of ingesting large quantities of organic waste.

Table 5. Comparison of some aspects of the biology of the vermicomposting species

	Eisenia fetida	***Eisenia andrei***	***Dendrobaena rubida***	***Dendrobaena veneta***	***Drawida nepalensis***	***Eudrilus eugeniae***	***Perionyx excavatus***	***Lumbricus rubellus***
Colour	Brown and buff bands	Red	Reddish purple	Reddish and purple bands	-	Reddish brown	Reddish brown	Reddish brown
Size of adult earthworms	4–8 mm × 50–100 mm (0.016–0.03 in × 1.9 × 3.0 in)	4–8 mm × 50–100 mm (0.016–0.03 in × 1.9 × 3.0 in)	3–4 mm × 35–60 mm (0.11–0.16 in × 1.3–2.4 in)	5–7 mm × 50–80 mm (0.2–0.27 in × 2–3.15 in)	-	5–7 mm × 80–190 mm	4–5 mm × 45–70 mm	4 mm × 70–150 mm
Mean weight of adults	0.55 g	0.55 g	0.25 g	0.92 g	0.82 g	2.7 – 3.5 g	0.5 – 0.6 g	0.80 g
Time to maturity (days)	28 – 30	21 - 28	54	65	34 - 42	40- 49	28 - 42	74 - 91
Nº of cocoons day^{-1}	0.35-0.5	0.35-0.5	0.20	0.28	0.15	0.42 - 0.51	1.1 - 1.4	0.07 – 0.25 mm (0.003 – 0.01 in)
Mean size of cocoons	4.85 mm × 2.82 mm (0.03 × 0.11 in)	4.8 mm × 2.82 mm (0.03 × 0.11 in)	3.19 mm × 1.97 mm (0.12 × 0.07 in)	3.14 mm × 1.93 mm (0.12 × 0.075 in)	-	-	-	3.50 mm × 2.46 mm (0.13 × 0.10 in)
Incubation time (days)	18 - 26	18 - 26	15 - 40	42.1	24	12 - 16	18	35 - 40
Hatching viability (%)	73-80	72	85	20	75-88	75-84	90	60-70
Number of worms cocoon^{-1}	2.5–3.8	2.5-3.8	1.67	1.10	1.93	2-2.7	1-1.1	1
Self-fertilization	+	+	+	-	+	-	-	-
Life cycle (days)	45-51	45-51	75	100-150	100-120	50-70	40-50	120-170
Limits and optimal T [a]	25°C (0°C – 35°C)	25°C (0°C – 35°C)	-	25°C (15°C – 25°C)	-	25°C (16°C – 30°C)	25°C – 37°C	-
Limits and optimal moisture	80% – 85% (70% – 90%)	80% – 85% (70% – 90%)	-	75% (65% – 85%)	-	80% (70% – 85%)	-	-

1.5.1. Intestinal microbiome of earthworms and interaction with exogenous microbiota

These invertebrates exert a strong stimulating effect on soil microorganisms through their continuous ingestion of soil (endogenetic earthworms) and mixed soil leaf (anecic earthworms). The intense excavating activity of these soil organisms contributes to dispersing microorganisms in the soil, thus increasing the process of decomposition of organic matter in the soil matrix. In addition, earthworms help increase soil fertility by forming an organic matter layer in the topsoil. These features, among others, have led to the popularity of earthworms as excellent bioindicators of soil pollution (Cortet et al., 1999; Lanno et al., 2004). Thus, earthworms are considered natural bioreactors because some microorganisms are proliferated in their guts, and they also provide suitable conditions for the biodegradation of waste (Munnoli et al., 2010). Earthworms produce nutrients for the microorganisms, creating a symbiotic and synergistic interaction (Aira et al., 2007; Hong et al., 2011) from their intestinal juice, or because of microbial activity, enzymes are generated. These intestinal enzymes capable of decomposing and biodegrading residues have been mentioned in several works of literature demonstrating their synergism (Sánchez-Hernández et al., 2009; Blouin et al., 2013). Thus, earthworms provide multiple environmental services in agroecosystems (Blouin et al., 2013). Of particular interest are the endogeic species, such as *Aporrectodea rosea*, which are numerically dominant in temperate agroecosystems (Whalen, 2006).

These organisms ingest large amounts of soil, or specific fractions of soil (i.e., organic matter), thereby being continuously exposed to contaminants through their alimentary surfaces (Morgan et al. 2004). Moreover, several studies have shown that earthworm skin is a significant route of contaminant uptake as well (Jager et al., 2003). Another earthworm species, vg. *Eisenia sp.,* can transform and stabilise biologically several raw organic fluxes in a vermicomposting process. These processes also have several beneficial trade-offs related to biomagnification and decontaminating capacities. Agulló et al. (2015) reported heavy metal accumulation in *E. fetida* earthworms, and Saez et al. (2021) observed a reduction of 50–75% of the recalcitrant polyphenols in olive-derived wastes after composting and vermicomposting.

1.5.2. Vermicomposting

The term vermicomposting was coined to describe the degradation of organic waste by earthworms. Loehr et al. (1984) proposed the word vermistabilization, which perhaps better defines the effect caused by the digestion of organic matter by earthworms. Another common

term is worm composting, which is considered by some authors to be a specific composting system.

The first reference to the benefits of vermicomposting, understood as the use of earthworms for the disposal of organic waste, was made by the Benedictine monk Augustus Hessing in the 1930s, when he used worms to dispose of the waste produced by the monastery (Blickwedel, 1983). However, knowledge of vermicomposting processes was initiated in the 1970s in the United States by professors Clive A. Edwards and E. Neuhauser. Edwards and E. Neuhauser (Cornell University) and R. Hartenstein (State University of New York, Siracuse) laid the scientific and technical foundations for the development of these systems. Subsequently, these processes have developed spectacularly in different countries in Europe (Great Britain, Italy, Holland, Spain), Australia, Africa (South African Republic), Southeast Asia (Philippines, China, India), and Central and South America (Cuba, Colombia, etc.) (Edwards, 1995).

Vermicomposting is a process of biooxidation and stabilisation of organic matter mediated by the combined action of earthworms and microorganisms, resulting in a product called vermicompost. This biotransformation practice makes use of several advantages derived from the activity of certain epigean species of earthworms, which accelerate the decomposition and humification of organic matter (Petrussi et al., 1988), either directly (detritivorous feeding and movement through galleries) or indirectly (stimulation of microbial activity). On the other hand, they improve the structure of the final product by breaking down organic materials, reducing their particle size, and favouring the formation of stable aggregates (Elvira et al., 1998).

Vermicomposting is a process by which biodegradable wastes, such as kitchen wastes, bio-wastes of agro-based industries, farm wastes, market wastes, and livestock wastes, passing through the worm gut, are transformed into nutrient-rich vermicompost. Earthworms are biological agents capable of consuming waste and depositing excreta in this process (Adhikary, 2012). This process involves a symbiotic interaction between some earthworms and microorganisms (Lim et al., 2012). Table 6 describes some investigations into the vermicomposting process.

In addition, the activity of these detritiviruses increases the nutrient content, converting them through microbial activity into soluble forms that can be assimilated by the crops (Edwards and Burrows, 1988). This process also favours the production of substances that can act with phytohormonal action on plants (Tomati et al., 1987). Finally, the vermicomposting process makes it possible to exploit earthworms as a source of protein for animal consumption (Sabine, 1988; Fisher, 1988).

Table 6. Vermicomposting process using different organic wastes.

Sector	Type of waste	Specie earthworm	Reference
Agri-food industry	Olive mill waste	*E. fetida & E. andrei*	Sáez et al (2021)
	Rice husk	*E. eugeniae*	Lim et al. (2012)
	Cereal crops	*E. eugeniae*	Suthar (2008)
	Mustard crop	*E. fetida*	Bansal & Kapoor (2000)
	Cotton crop	*E. fetida*	Albanell et al., (1988)
	Coffee pulp	*E. fetida*	Orozco et al., (1996)
	Olive mill waste	*E. andrei*	Melgar et al., (2009)
	Wine industry	*E. andrei*	Nogales et al., (2005)
	Sugar cane	*E. eugeniae & L. mauritii*	Parthasarathi & Ranganathan (1998)
Chemical industry	Petrochemical sludge	*E. fetida*	Martín-Gil et al., (2008)
	Prestige tar	*E. eugeniae*	Rajesh Banu et al., (2005)
Livestock farming	Livestock waste	*E. fetida*	Agulló et al., 2015
	Livestock waste	*P. excavatus*	Kale et al., (1982)
	Livestock waste	*E. fetida*	Chan & Griffiths (1988)
	Livestock waste	*E. fetida*	Hamilton et al., (2008)
Sewage water	Urban sewage sludge	*E. fetida*	Frank et al., (1983)
	Dairy industry sludge	*E. fetida*	Nogales et al., (1999)
	Industrial sludge from paper mills	*E. fetida*	Elvira et al., (1996)
	Non-recyclable industrial paper sludge	*E. fetida*	Gupta & Garg (2009)
	Fish farm effluent sludge	*E. fetida*	Marsh et al., (2005)
	Biogas production sludge	*L. mauritii & E. fetida*	Tripathi & Bhardwaj (2004)
	Cane distillate sludge	*P. excavatus*	Suthar & Singh (2008)
Textile industry	Textile mill sludge	*E. fetida*	Garg et al., (2005)
	Textile mill sludge	*E. fetida*	Kaushik & Garg (2003)
	Textile mill sludge	*E. andrei*	Nogales et al., (1998)
Urban	Plant waste from gardening	*E. andrei & L. rubellus*	Engelstad (1991)
	Plant waste from gardening	*P. excavatus*	Manna et al., (1997)
	Plant waste from gardening	*P. excavatus*	Reddy & Ohkura (2004)
	Solid Urban Waste	*E. eugeniae*	Aalok et al., (2008)
	Organic kitchen waste	*P. excavatus*	Chaudhuri et al., (1993)
Others	Kitchen waste, agro-residues & industrial textile waste	*E. fetida*	Garg et al., (2006)

Vermicomposting represents a clean technology with no environmental impact and reasonably moderate investment, energy, and maintenance costs. Its use can be summarised into three concepts: a) transformation of organic waste that can be harmful, unhealthy, or annoying; b) generation of a high-value biofertilizer (called vermicompost), of great value as an amendment that works well as a chemical-organic fertiliser; c) production of a large biomass of earthworms, of high protein content and of high quality for animal feed (mainly pigs, poultry, and fish).

These diverse functions create a relatively new economic activity called "vermicomposting". From the cultivation of earthworms in the open air or under cover (Edwards, 1995; Rivero, 1993), two sources of income are derived: vermicompost as a high-quality organic fertiliser, the leachates generated as liquid organic fertiliser (Benítez et al., 1995), and earthworms as protein for animal consumption.

Since organic matter acts as a substrate and food in vermicomposting and the soil is not involved, only epigeic earthworms can be used in the process. Only five species of worms are widely used in vermicomposting: *Eisenia andrei* (Savigny), *Eisenia fetida* (Bouché), *Dendrobaena veneta* (Savigny), and, to a lesser extent, *Perionyx excavatus* (Perrier) and *Eudrilus eugeniae* (Kinberg). *E. fetida* and *E. andrei* are the species most widely used in vermicomposting and vermiculture facilities worldwide.

Vermicompost is represented by earthworm excreta, which are able to enhance nutrients and the status of soil health. It is considered a cost-effective biotreatment to convert organic waste into stabilised humic products (Sáez et al., 2021). Vermicompost enhances soil fertility not only biologically but also physically and chemically. Physically, the treated soil has better bulk density, aeration, porosity, and water retention. Chemically, electrical conductivity, pH, and organic matter content are enhanced, leading to better crop yield (Lim et al., 2015). In fact, the abuse of inorganic fertilisers without organic supplements deteriorates the chemical and physical properties of land and pollutes the surrounding environment (Manivannan et al., 2009). It was observed that the addition of vermicompost (20 t/ha) to agricultural soil over two consecutive years significantly improved aggregate stability and soil porosity (Bouajila and Sanaa, 2011), improved the availability of air and water, and encouraged root growth (Gopinath et al., 2008). Summarising, the epigeal earthworm *Eisenia fetida* has been widely used due to its great capacity to decompose organic matter. Apart from its widespread use in vermicomposting throughout the world, *Eisenia fetida* has also been used as an ecotoxicological model (bioindicator) in the soil (accepted by the scientific community) due to its easy reproducibility and maintenance under laboratory conditions.

1.6. Ecotoxicological response to stressing factors and acclimation to the presence of agroplastics in vermicomposting process

Earthworms play an important role in many terrestrial ecosystems as ecosystem engineers but also as important bioindicators of media quality. In this line, earthworms play a significant role in the risk assessment of potential polluting substances as long-used model species in regulatory ecotoxicology. Their functional significance, i.e., their effects on ecosystem process, makes earthworms important study organism in ecological and eco-toxicological research (Latif et al., 2013; Lubbers et al., 2013). These species are model organisms in ecotoxicology, and they play a significant role in contaminant fate, are recommended test organisms according to international standards such as International Organization for Standardization (ISO) and Organization for Economic Cooperation and Development (OECD) (ISO 11268-1:2012; OECD, 2016). The *Eisenia fetida* is specie that are considered as bioindicator of pollution. The use of *Eisenia fetida* as a bioindicator susceptible to contaminants in vermicompost remains open to debate in the scientific community, as it is often less susceptible to contaminants than other earthworm species and is also rarely found in agricultural soils (Spurgeon et al., 2003). The earthworm toxicity test is a European test (OECD guideline 207) in which earthworms are exposed to compost in varying amounts. Following 14 days of exposure, the number of surviving earthworms is counted and weighed, and the percent survival rate is calculated. In the acute toxicity test, earthworms are exposed to high concentrations of the test material for short periods of time. Hou et al. (2013) highlighted in their review that invertebrates such as the earthworm *Eisenia fetida* can be used to quantitatively assess the extent of bioaccumulation by calculating the ratio of MNO tissue concentration to that in water (bioaccumulation factor or BCF), and biomagnification by the ratio of the MNO concentration in the predator to that in its prey (biomagnification factor or BMF). Furthermore, the biota-sediment accumulation factor (BSAF) represents the ratio of the MNO concentration in an organism to that in the sediment (Hou et al., 2013).

There are different factors that produce an ecotoxicological response in earthworms vg. presence of heavy metals, xenobiotics, pesticides, plastic waste etc. The plastic waste, they can produce a direct effect / ingestion (abrasion, inflammation, and other symptoms) (Wright et al., 2013), will be produced an accumulation in their tissues, and an indirect effect / contact (development of oxidative stress). Toxicity can also arise from leaching constituent contaminants, such as monomers of plastics additives, capable of causing carcinogenesis (Wright et al., 2013). They are also applied as bioindicators to assess the quality of vermicompost and for the assessment of the contamination degree and toxicity of chemical

contaminants (Brulle et al., 2010; Nahmani et al., 2007; Rombke et al., 2006), as in survival trials.

The distribution of microplastics is affected according to their sources, which are defined as primary (e.g., exfoliating cleansers, body washes, toothpastes, cosmetics, packed beds of air-blasting scrubbers, post-industrial manufacturing plastic scrap) and secondary (e.g., as a result of chemical and biological deterioration in the environment) (Fendall and Sewell, 2009; Browne et al., 2011; McCormick et al., 2014; Wang et al., 2016). The physico-chemical properties of microplastics, such as size, shape, density, colour, and chemical composition, greatly affect their transport in the environment and their bioavailability (Wright et al., 2013).

Exposure to microplastics leads to a wide range of physiological alterations, mainly to the digestive and immune systems (Sharifinia et al., 2020; Trestrail et al., 2020). Ingestion of plastic fragments can cause direct adverse effects such as obstruction of the digestive canal, abrasion and destruction of the epithelium, and cell lysis. Adverse effects will depend on the type, size, and shape of the fragments. It can also generate indirect effects such as inflammation, oxidative stress, and metabolic alterations.

Nanoplastics are an emerging contaminant of concern that is closely related to microplastics. The largest source of microplastics originates from microplastic or mesoplastic objects that are unintentionally released into the environment (e.g., by littering) and break down to secondary microplastics (Andrady, 2017; Qi et al., 2020), with continuous weathering that eventually generates nanoplastics (Gigault et al., 2018). Most research to date has focused on microplastics with particles >10 μm in size, largely due to the resolution limits of the analytical equipment that is used to identify and quantify these materials (Shim et al., 2017). As such, there are a limited number of papers investigating nanoplastics and earthworm species; notably, the use of traceable materials such as fluorescently labelled polystyrene (PS) beads enable detection with higher special resolution through the use of fluorescence microscopy.

Jiang et al. investigated *E. fetida* exposure to micro- (diameter 1300 nm) and nanoplastic polystyrene particles (diameter 100 nm) at 1000 μg kg^{-1} (Jiang et al., 2020). In general, the results show that the toxicity of micro- and nanoplastics in *E. fetida* is quite low. However, particle accumulation, which was four times larger for microparticles than for nanoparticles, was observed. In addition, the authors used a comprehensive set of physiological and biochemical endpoints and concluded that microplastics were more toxic than nanoplastics.

Importantly, research to assess nanotoxicological effects on terrestrial invertebrates is still needed (Johnson et al., 2018; Mukherjee and Acharya, 2018), particularly given that the chemical transformation of nanoplastics in soils presents a high level of complexity and

consequently, organic/organo-mineral composition under the influence of nanoplastics renders an unpredictable event.

Overall, the available literature on the hazardous effects of nanoplastics in earthworms is limited and indicates that hazards are mainly driven by mechanical damage to the intestine. Importantly, larger microscale particles appear to be more hazardous than nanoscale plastics, as determined by this endpoint. However, a range of other potential toxicity mechanisms are possible for nanoplastics in earthworm species, including toxicity caused by the leaching of plastic additives that could exert additional negative impacts after exposure.

In summary, the mechanisms, and potential toxic effects of microplastics and nanoplastics reported for earthworms produce the following effects at five different levels: at the organism, organ, cellular, biochemical, and genetic levels. At the organism level, exposure can cause changes in end points, such as avoidance, survival (even death), growth, and locomotion. At the organ level, abnormalities in the dermal and intestinal pathways and barriers have been reported by histopathological observation. At the biochemical level, impacts on different stress pathways have been reported, including general stress and oxidative stress (ROS). These responses could activate signalling cascades that modulate key genetic markers.

1.7. Biodegradation capabilities of epigeic earthworms

The biotic degradation then must be initialized by ageing or processes that reduce crystallinity and packaging of polymers to obtain hydrolysable plastics (able to be degraded under the action of exocellular enzymes, and therefore the degradation products (monomers) be absorbed into the microorganism cell to be used as metabolic substrate and finally plastic derived chemical elements, used into the microorganism tissue, or emitted as CO_2 or H_20.

A metagenomic analysis to characterize the bacterial communities of casts from *E. andrei* fed with different food sources found that the bacterial communities of cast strongly depended on the food source ingested by earthworms (Aira et al., 2016). These promising results encourage the use of earthworms in the biodegradation of AWP. This novel approach would increase the potential use of these organisms to manage plastic waste by farmers.

The main limitations for the biodegradation of plastics include the high molecular weight of polymers, the lack of favourable functional groups, and the high degree of crystallization. Therefore, long-chain polymers cannot cross the cell membrane and must be treated with extracellular enzymes (Wilkes and Aristilde, 2017). Synthetic plastics tend to be highly hydrophobic as they present stable functional groups such as alkane and phenyl.

Consequently, oxidation and hydrolysis are necessary steps to increase hydrophilicity and promote microbial attack (Shah et al., 2008). Likewise, plasticizers and additives significantly influence the biodegradability of polymers. Therefore, the degradation of microplastics consists of an event composed of various physical-chemical and microbial factors that occur naturally in the environment (Urbanek et al., 2018). In this way, microplastics can act as constituents of an ecological niche that allows the colonization and growth of organisms, offering an important source of carbon to use (Yuan et al., 2020). However, this capacity is only available for those organisms that have enzymes capable of incorporating the products obtained, monomers of plastic polymers, in the corresponding metabolic routes for obtaining energy (Pham et al., 2021).

In Figure 11 shows a hypothesized model on plastic biodegradation by vermicomposting technology, which is described as a two-step process; 1) the cast-associated process, where the microorganism in earthworms cast and other decomposer fauna (Collembolans) actively participate in further decomposition of organic matter content. 2) The gut-associated process where the fresh organic matter ingested by earthworms undergoes physical and biochemical transformation that are mediated by enzymes released to the luminal space by both, symbiont microorganism and the earthworms gut epithelium.

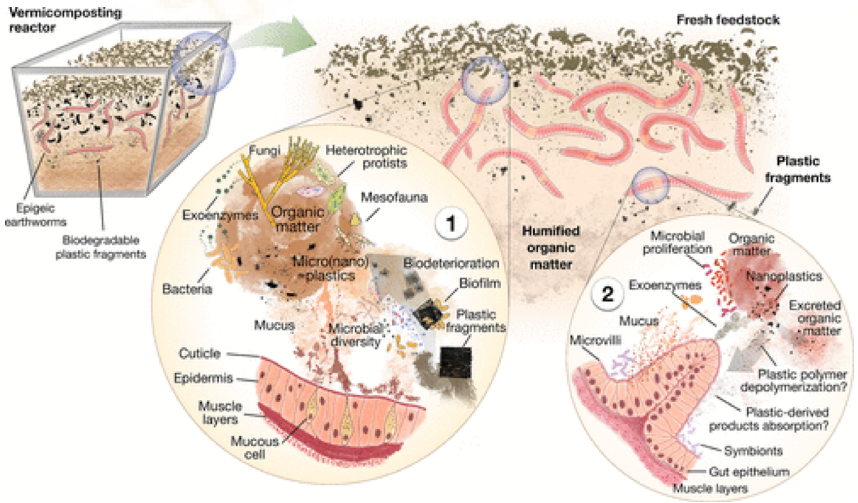


Figure 11. Hypothesized model on plastic degradation in vermitechnology extracted from (Sánchez-Hernández et al., 2020a)

2. Objectives

2. Objectives

The main objective of this PhD thesis is to study how the presence of plastics in organic waste fluxes affects the vermicomposting process as valorization treatment, using *Eisenia fetida (EF)* earthworms as the main indicator and also how different approaches (ageing-pretreatments, beneficial microbiome) can reduce EF affection by plastics and potentially also contribute to plastic degradation during the vermicomposting.

The specific objectives of the research are:

- *Objective 1:* Know the main the types of plastics in the organic waste fluxes (AWP) used for vermicomposting and the potential contribution of abiotic pre-treatments in facilitate their degradation (Paper 1).
- *Objective 2:* Determine the survival, morphological and ecotoxicological effects of AWP on *E. fetida* (Paper 2 and 3).
- *Objective 3:* Analyse the effect of AWP on the vermicomposting process and the resulting quality of the vermicompost obtained (Paper 2 and 3).
- *Objective 4:* Analize the natural microbiome of *E. fetida* and identify their variation in AWP polluted media to extract, cultivate, and use beneficial EF microorganisms as prebiotics in vermicomposting (Paper 4).
- *Objective 5:* Produce and validate an optimized vermicomposting procedure integrating AWP pre-treatments and beneficial microorganisms to improve vermicomposting in AWP polluted conditions (Paper 4).

Different studies have been carried out to achieve these objectives, collected in the following four publications:

- **Paper 1:** Plastic waste treatments: efficiency of the degradation processes. ***Polimers***, *2024 (accepted, in press).*
- **Paper 2:** The effects of agricultural plastic waste on the vermicompost process and health status of *Eisenia fetida.* ***Agronomy****, 2022 (https://doi.org/10.3390/agronomy121025472023).*
- **Paper 3:** Effect of agricultural microplastic and mesoplastic in the vermicomposting process: Response of *Eisenia fetida* and quality of the

vermicomposts obtained. ***Environmental Pollution***, *2023 (https://doi.org/10.1016/j.envpol.2023.122027).*

- **Paper 4:** Unlocking the biotechnological and ecotoxicological perspectives of microplastic degradation by means *Eisenia fetida* inoculated with polymer degrading capabilities microorganism consortia. ***Environmental Pollution***, *(under review).*

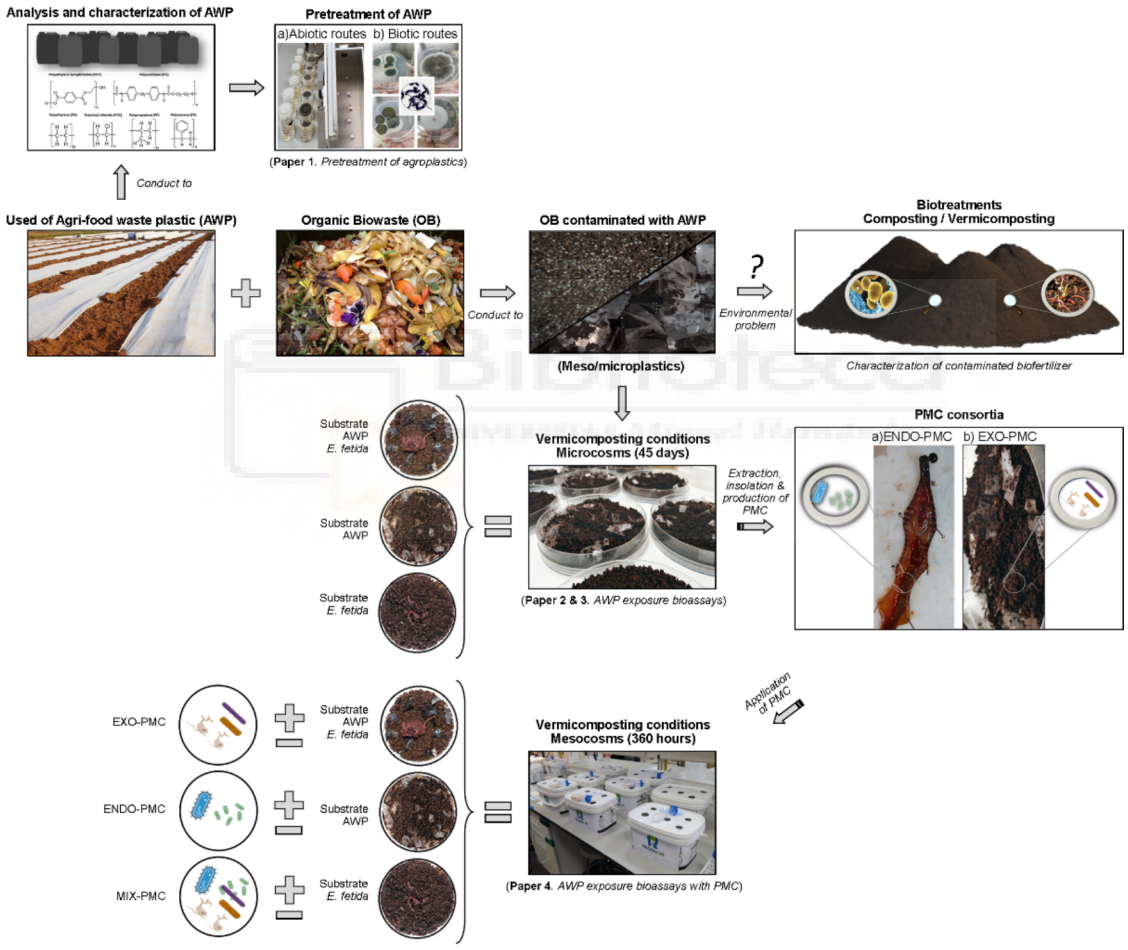


Figure 12. Correlation between the main objectives and the papers included in the thesis.

3. Material and Methods

3.1. Plastic waste inventory selection

In this thesis we are mainly focus in the effects of plastics on vermicomposting. The main way of contact of *Eisenia fetida* with plastics is related to the presence of plastics in organic waste fluxes, specially from organic waste collection and agrofood wastes. In general terms, the most common polymers used in agrofood and agriculture are polyethylene (PE) and polypropylene (PP). Polyethylene exists in two main forms, namely high-density polyethylene (HDPE) and low-density polyethylene (LDPE), but we can also mention linear low-density polyethylene (LLDPE) (Scarascia-Mugnozza et al., 2011). Concerning food packaging plastic waste, the five most common polymers used are PE (including LDPE, LLDPE, and HDPE), PP, PS, PET, and PVC.

Using this assessment, a specific collection of plastics was developed, and the main characteristics of the plastic samples are summarized in Table 7. Four different plastic materials were considered for this thesis: low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), polyethylene terephthalate (PET), polystyrene (PS), LLDPE + LDPE and Expanded Polystyrene (EPS).

Plastics (MP) of selected samples was milled using specific milling equipment, and the powdered samples (with the specific size profile) were used along the all experiments (Figure 13). A set of each milled plastic was also pre-treated with different strategies (section 3.1).

Table 7. Main characteristics of the plastic samples used.

AWP type	Origin	Film Size/ Thickness	Number of MP items / 100 mg	% Fraction size of MP in weight (mm)				
				>2	1-2	0.2-1	0.05-0.2	<0.02
LDPE	Solplant	1cm^2/20 µm	137±16	7.5%	78.7%	16.2%	0.3%	0.2%
LLDPE	Repsol	1cm^2/50 µm	352 ±43	25.7%	61.1%	14.4%	0.3%	0.6%
PET	UNIPI	1cm^2/200 µm	247±28	30.6%	53.2%	14.0%	2.4%	1.9%
PS	UNIPI	1cm^2/200 µm	284±32	6.4%	73.4%	17.5%	5.0%	1.1%
LLDPE + LDPE	Solplant	1cm^2/50 µm	248±18	18.8%	68.5%	13.0%	0.3%	0.5%
EPS	UMH	1cm^2/1280 µm	-	-	-	-	-	-

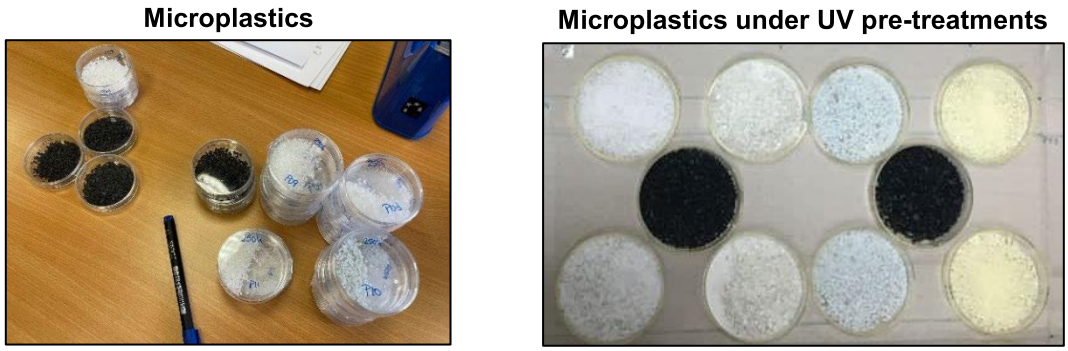


Figure 13. Detail powders produced by OWS.

3.2. Procedure for abiotic pre-treatment of plastics

We selected significant pre-treatments to enhance plastic degradation, grouped by the main treatment types: a) Thermal treatments, b) Photo-oxidation treatments, c) Chemical/thermochemical treatments and d) e-beam.

a) Thermal treatments: the proposed treatments are linked to the exposition of plastics to different ranges of temperature, combined or not, with other degrading agents (mainly chemical attack).

   Thermal gravimetric analysis (TGA) was performed using a Q500 thermogravimetric analyser produced by TA Instruments, under nitrogen atmosphere, on about 2 mg of sample, from room temperature to 700 °C with a heating rate of 10.00 °C/min.

b) Photo-oxidation treatments: the proposed treatments are based on the exposure of plastic to different UV ranges and durations. Selected UV ranges were UV-A, UV-B and UV-C, in accordance with the literature.

   The plastic samples (4 cm^2) were submitted to different range of exposition time and different wavelengths (UV A: 350 nm, UV B: 320 nm and UV C: 253 nm). A detail of the UV chambers (A, B and C) used as shown in Figure 14.

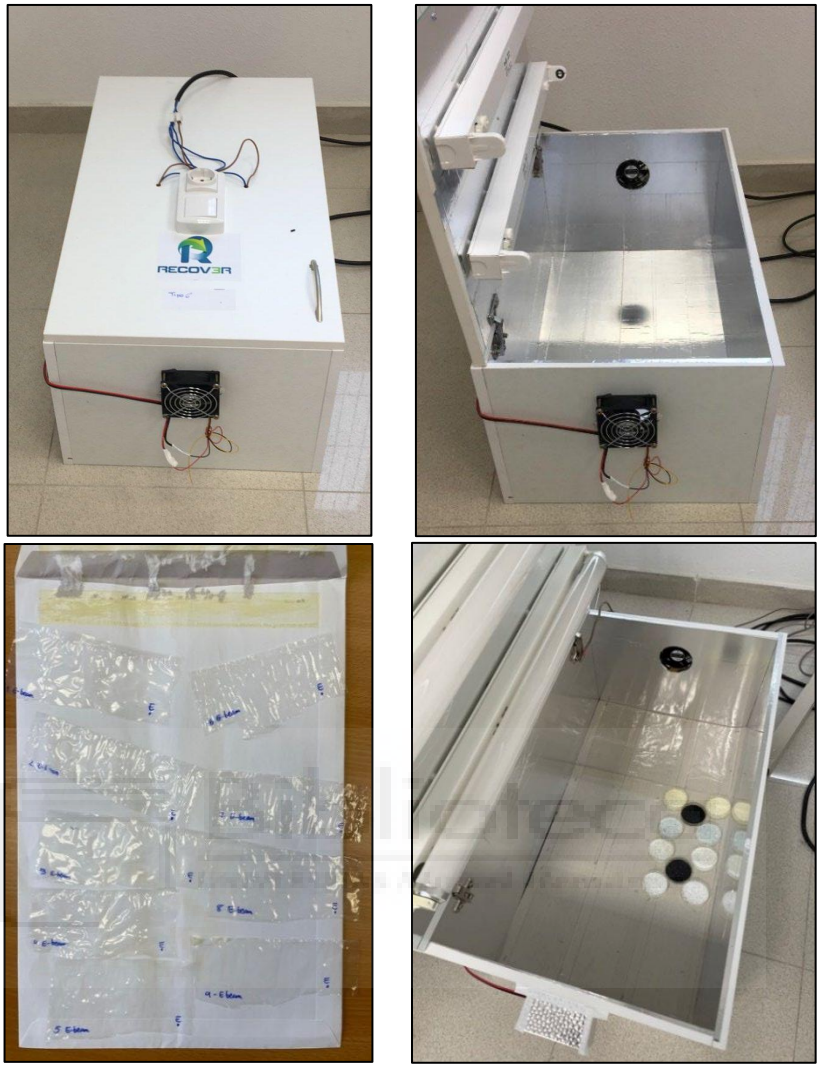


Figure 14. Detail of the UV chambers (A, B and C) used

Table 8. UV chamber characteristics

Type UV	W x chamber	Irradiated surface (m^2)	Power consumption (kW/m^2 h)
A	18	0.2479	0.145
B	20	0.2479	0.161
C	16,7	0.2479	0.135

c) Chemical/thermochemical treatments: the proposed treatments are based on the exposure of plastic to several oxidising agents, including extreme pH conditions in different conditions of reaction temperature, attack duration, and oxidizer concentration. In Table 9 showed the used chemical treatments.

Table 9. chemical treatments.

Treatments	Plastic type	Conditions
$HCl:HNO_3:H_2O$ 3:1:1, Aqua regia	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	7 & 14 days, 25ºC, 35 & 70ºC, concentrated and diluted (1 to 3) reagents
$K_2Cr_2O_7 + H_2SO_4$	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	7 & 14 days, 25ºC, 35 & 70ºC, concentrated and diluted (1 to 3) reagents
$(NH_4)_2S_2O_8 + H_2SO_4$	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	7 & 14 days, 25ºC, 35 & 70ºC, concentrated and diluted (1 to 3) reagents
H_2O	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	7 & 14 days, 25ºC, 35 & 70ºC

Figure 15. On the left: Used chemical reagents. On the right: chemically treated plastics.

d) E-beam pre-treatment: Plastics were exposed also to irradiation by e-beam in different conditions of energy irradiation (100–1000 kGy). An EBlab 200 device (Comet, Switzerland), specialized for low energy electron beam treatment; harboring an EBA-200 e-beam lamp operating at accelerating voltage from 70 to 200 KeV which correspond to a total dose energy from 100 to 1000 KGy, was also evaluated for polymer degradation. The analyses were carried out in ambient atmosphere. Plastic samples were as plastic probes with maximum dimensions of 21 cm x 30 cm.

Figure 16. e-Beam instrument used for pre-treatment of plastics

3.3. Analytical approach to evaluate plastic degradation

In our thesis, the main techniques used in the assessment of the effects of pre-treatments on the selected plastics are A) visual inspection, B) thermal analysis, especially thermogravimetric analysis (TGA) and C) spectroscopic methods, especially infrared spectrophotometers.

The evaluation of the initial effectiveness of treatments was obtained using visual parameters. After all, FTIR analyses were performed to establish changes in plastic structure and composition at the surface scale, especially in PE-derived plastic samples. Finally, to confirm the above effects, specific thermal analyses were developed to study changes in crystallinity and other key parameters.

A) Visual parameters

In all the samples, a visual checklist of visual parameters was done in order to establish a qualitative classification of treated vs. non-treated plastic. The list of visual parameters is: gloss retention, surface discoloration, plastic yellowing, crazing, surface roughness, curling or enrolling and hand fragmentation.

In each of these visual parameters, we use the qualitative scale represented in Table 10.

Table 10. Qualitative scale of visual parameters.

Evidence	Qualitative Scale
No visible effect	0
Low effect	1
Medium effect	2
High visible effect	3

The evaluation of these visual parameters using the previous qualitative scale allowed us to develop a preliminary integrated classification of the pre-treatment effects, which were verified with the other techniques. Figure 17 shows an example of the visual changes caused by the exposure of plastics to UV pretreatment.

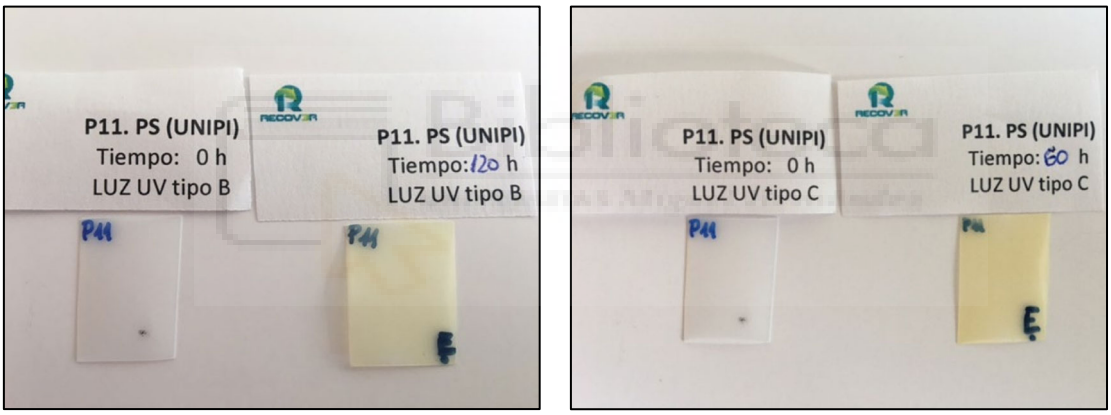


Figure 17. Example of visual changes after exposition to UV pre-treatments.

B) Infrared Spectrophotometry

When infrared radiation strikes a sample, it can cause changes in the vibrational states of the constituent molecules of the sample. The absorption of radiation by a sample is indicative of the type of links and functional groups present. Thus, this technique is used in material characterization by examining the existing bonds in a sample. It works by searing different beams of varying frequencies and measuring the absorption of the beam by the sample. Each bond in a sample has a different energy and angle, thus absorbing the beam at specific wavelengths. The raw data collected from the different beams is modelled using the technique of Fourier transform, which allows a mathematical operation to present the measurement as absorption per wavelength. This

allows the measurement of a wider range of wavelengths with high resolution (Al-Salem, 2018).

From an instrumental point of view, both of its applications make it convenient to divide the infrared region into three regions called near-infrared (NIR), mid-infrared (MIR), and far-infrared (FIR). Analytical infrared spectroscopy: the vast majority of applications are based on the use of middle infrared (4000-600 cm^{-1}) and near-infrared, which provides the possibility of converting this technique into a quantitative technique.

Thus, FTIR allows for the monitoring of the evolutions in the chemical structure of a polymer due to the formation or disappearance of functional groups during the process of degradation (Sudhakar, Doble, Murthy, and Venkatesan, 2008; Shi et al., 2019).

In this section, several instruments were used to characterize plastics and polymers:

- Spectrometer BRUKER IFS 66/S, able to work with a resolution of up to 1 cm^{-1}. It has a source, a KBr beam splitter, and two detectors: A DLaTGS detector at room temperature for routine measurements of routine and obtaining spectra between 7000 and 400 cm^{-1} and another MCT detector cooled with liquid nitrogen for high sensitivity and speed of measurement, ideal for action kinetics between 600 and 4000 cm^{-1}.
- JASCO FTIR 4700 spectrometer, able to work with a resolution of up to 0.5 cm^{-1}. It has a source of go media, a beam splitter KBr encapsulated in germanium, and a detector DLaTGS for routine measurements. The range of measurable frequency is between 7800 and 400 cm^{-1}.

From the data obtained from the FTIR spectra of the plastic samples, it is also possible to determine several indexes reported in previous works to assess the degradation of the polymer (Martinez-Romo et al., 2015; Gupta et al., 2020; Shi et al., 2019; Chen et al., 2021). These indexes are defined as the ratio between the integrated band absorbance of the functional group (or specific bond type), which may result from polymer degradation, and that of a reference peak used to characterize the degree of oxidation of the considered polymer:

$$Functional\ group\ index = \frac{Absorbance\ functional\ group\ peak}{Absorbance\ reference\ peak}$$

According to the values obtained in these indexes, the higher the values, the greater the degree of degradation of the polymer. As an example, one of the most commonly used is the carbonyl index (CI), often used as an indicator of the presence of carbonyl groups, which may result from polymer degradation (Rodrigues et al., 2018; Prata et al., 2020). Considering the previous works that include the use of these indexes to estimate plastic degradation, we have defined an adaptation of these indexes based on the FTIR response obtained and the type of polymer studied.

C) Thermal properties with TGA

Thermal gravimetric analysis (TGA) was performed using a Q500 thermogravimetric analyzer produced by TA instruments under a nitrogen atmosphere on about 2 mg of sample from room temperature to 700 °C with a heating rate of 10.00 °C/min.

Spectral images were acquired in μATR mode using the infrared imaging system Spotlight 300 (Perkin Elmer). The spectral resolution was 4 cm^{-1} with a spatial resolution of 100 x 100 μm. Background scans were obtained from a region without a sample. IR images were acquired with a liquid nitrogen-cooled mercury-cadmium telluride line detector composed of 16-pixel elements. Each absorbance spectrum composing the IR images, resulting from 16 scans, was recorded for each pixel in the μATR mode using the Spotlight software. The spectra were collected by touching the ATR objective on the sample and collecting the spectrum generated from the surface layer of the sample. The Spotlight software used for the acquisition was also used to pre-process the spectra. IR spectral images were produced by using the absorbance in each frequency range, 4,000–720 cm^{-1}. Spectra contained in the spectral images were analysed using a comparison reference spectrum. The obtained correlation map indicates the areas of an image where the spectra are most like a reference spectrum.

The specific areas of interest were identified by means of the optical microscope, the ATR objective was touched on the sample, the spectra generated from the surface layers of the sample were collected, and IR spectral images were produced. The recorded spectral maps were elaborated using the software of the instrument to obtain a correlation map. The acquired chemical map was used to obtain the average spectrum, which is the most representative spectrum of the chemical map (reference spectrum). The correlation map between the chemical map and the average spectrum was then elaborated using the instrument software to investigate the chemical homogeneity of the

sample. A correlation index closes to 1 on the entire analysed sample was considered indicative of the chemical homogeneity of the sample.

3.4. Selection of earthworms with potentially degrading capabilities in vermicomposting processes

We selected earthworm species that will work best in plastic polluted environment. We used two main criteria for this selection: 1) Evidence of AWP interaction, assessed by literature studies on real experiments on AWP-earthworm systems, including survival and/or morphological effects; and 2) Usability, based on the abundance of specific information related to and the general usability of each earthworm species (Table 11).

Table 11. Usability conditions for each earthworm species.

General conditions	Epigeic species	AWP interaction evidence	Usability range*
TOM>30% f.w. Water content >50% f.w.) APW: 1.5% f.w. (0.45% d.w.)	• *Allolobophoridella eiseni*	• No	• 1
	• *Bimastus eiseni*	• No	• 1
	• *Bimastus minusculus*	• No	• 1
	• *Dendrobaena attemsi*	• No	• 1
	• *Dendrobaena hortensis*	• No	• 1
	• *Dendrobaena octaedra*	• No	• 1
	• *Dendrobaena veneta*	• No	• 1
	• *Dendrodrilus rubidus*	• No	• 1
	• *Drawida modesta*	• No	• 1
	• ***Eisenia andrei***	• **Yes**	• **3**
	• ***Eisenia fetida***	• **Yes**	• **3**
	• *Eiseniella tetraedra*	• No	• 1
	• ***Eudrilus eugenie***	• **Yes**	• **2**
	• *Helodrilus oculatus*	• No	• 1
	• *Lumbricus castaneus*	• No	• 1
	• *Lumbricus festivus*	• No	• 1
	• *Lumbricus friend*	• No	• 1
	• ***Lumbricus rubellus***	• **Yes**	• **3**
	• ***Metaphire californica***	• **Yes**	• **2**
	• *Perionyx excavatu*	• No	• 1
	• *Satchellius mammalis*	• No	• 1

**Usability range: 1 no reference, 2 some reference, 3 commercial use*

Regarding this environment and organic waste fluxes polluted by plastics, the epigean earthworms *Eisenia andrei*, *Eisenia fetida,* and *Lumbricus rubellus* achieved the highest usability, followed by *Eudrilus eugenie* and *Metaphire californica*. The commercial availability of *Eisenia andrei* and *Eisenia fetida* to validate their usability compared to *Lumbricus rubellus* is significantly higher, and therefore these two species were mainly

selected. Finally, *E. fetida* was selected for this study because this epigean species is recommended in several standard ecotoxicological tests (OECD 2016) for use in the assessment of various contaminants.

3.5. Design and development of normalized vermicomposting bioassays to study the effects of AWP presence

3.5.1. Experimental design

The environment used was an organic waste flux polluted by plastics. These media are normally associated with mixed AWP collection or mechanical-biological treatments of municipal wastes, where impurities of plastics are increasing. This waste flux is normally rich in organic matter (TOM > 30% f.w.) and humidity (water content >50% f.w.). *Epigeic* earthworms used to live in these environments traditionally in vermicomposting processes. The Regulation (EU) 2019/1009 of The European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003 said: “The compost shall contain: (a) no more than 6 mg/kg dry matter of PAH 16 (5); (b) no more than 3 g/kg dry matter of macroscopic impurities above 2 mm in any of the following forms: glass, metal, or plastics; and (c) no more than 5 g/kg dry matter of the sum of the macroscopic impurities referred to in point (b). From July 16, 2026, the presence of plastics above 2 mm within the maximum limit value referred to in point (b) shall be no more than 2.5 g/kg dry matter. By July 16, 2029, the limit value of 2.5 g/kg dry matter for plastics above 2 mm shall be re-assessed to consider the progress made with regards to the separate collection of biowaste. Then, the range expected to be treated will be 2-3% of AWP expressed as fresh weight. Thus, the presence of AWP plastics in mesocosm assays with epigeic earthworms will be fixed in this range to consider the remaining material as compost fertilizer. Considering a water content of 70% in *E. fetida* assays, the established value of 1.25% f.w. is equivalent to 0.45% d.w.

The exposure bioassay was designed to reproduce the conditions of the vermicomposting process of agro-industrial organic wastes with plastic presence at laboratory scale. For this reason, the different AWP materials (LLDPE + LDPE and EPS, for the second paper; LDPE, LLDPE, PET, PS, and LLDPE + LDPE, for the third paper), pre-treated or not with UV light, and at different sizes (mesoplastic and microplastic), were used (for the third paper).

3.5.2. Experimental set-up

The earthworms used in this study belong to the species *Eisenia fetida* and were obtained from large rearing containers (250 litres) kept under controlled conditions (20° ± 2 °C and darkness). The earthworms were fed with the same feedstock used in the laboratory bioassay for 30 days to favour their adaptability. For the development of the lab bioassay, adult citellated earthworms were selected with a body mass weight between 250mg and 600 mg (Figure 18), as recommended by international guidelines (OECD 2016). Considering the main factors established in the experiment (plastic type, pre-treatment or not, particle size, *E. fetida* presence, and AWP presence), three different types of experimental devices with three replicates each were prepared: a) feedstock + AWP without earthworms; 2) feedstock + earthworms without AWP presence; c) feedstock + APW + earthworms.

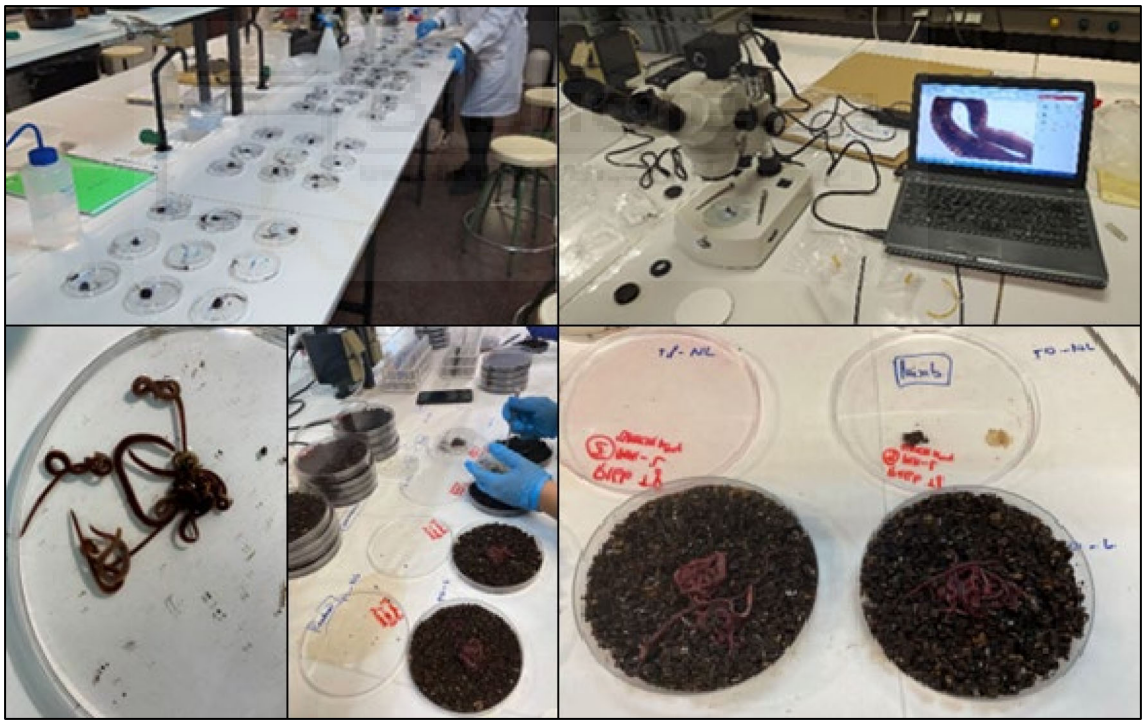


Figure 18. Vermicomposting bioassay and selection of earthworms.

The bioassay consisted of an incubation for 45 days in Petri dishes (15 cm ø) (Dominguez, 2018) using 80g of feedstock adjusted with distilled water to 70% moisture content. Then, 1g of APW material was added per replicate (1.25 % f.w. proportion) (Sáez et al., 2022). The incubation containers were kept in isolated chambers under controlled conditions (20° ± 2 °C and darkness). After 7, 21, 30, and 45 days of exposure,

survival and body weight variation were determined. For this, earthworms were carefully extracted from the Petri dish of each replicate by hand sorting, counted for survival, weighted, and recorded in order to obtain the mean body weight of each treatment. During the exposure time of the bioassay, when mortality was observed, the earthworms were immediately removed from the Petri dish. In addition, at the end of the bioassay, the final materials obtained, after the bioassay without earthworms (hereinafter referred to as substrate) and with earthworms (vermicompost) from each replicate, were homogenised and the samples were divided into two subsamples: one was used immediately for moisture determination and frozen at -80°C until enzyme activity determination, and the other was partially air dried in an oven equipped with forced aeration at 60 °C. To obtain a dust particle size, the sample was ground using an agate ball mill (RESTCH Mod. MM400) and dried at 105ºC for further determinations.

A single compost was used as substrate in all the assays (Table 12). This compost was developed in the COMPOLAB-UMH facility at a commercial-pile scale (10 m^3) using four ingredients (agri-food sludge +cow manure + vineyard pruning, 45 + 15 + 40 in volume %). The composting process lasted 96 days, including four turning events. High quality standards were achieved in terms of stabilisation, sanitization, and the absence of phytotoxicological effects. Heavy metals also accomplished Class A fertiliser regulation.

Table 12. Characteristics of the feedstock used.

Physicochemical parameters					Macronutrients				Mature parameters		
pH	EC (dS m^{-1})	BD (g L^{-1})	TOM (%)	TOC (g kg^{-1})	TN (%)	P (%)	K (%)	GI (%)	C_{HA} (%)	C_{FA} (%)	CEC (meq $100g^{-1}$ MO)
7.8	4.5	486	63.0	225	2.13	0.41	1.01	108	1.93	2.27	128

EC: Electrical conductivity, BD: Bulk density, OM: Organic matter, TOC: Total organic carbon, TN: Total nitrogen, GI: Germination index, C_{HA}: Acid humic-like carbon, C_{FA}: Acid fulvic-like carbon, CEC: Cation exchange capacity.

3.5.3. Analytical methods

Physico-chemical and chemical characteristics of the substrate and vermicompost

After homogenization, each vermicompost sample was divided into two subsamples. One was used immediately to determine the moisture content, and the other subsample was frozen at −80ºC to monitor the enzyme activity, and the other was kept at 45ºC in an oven with forced aeration to dry. This subsample was then ground to obtain dust

particles using an agate ball mill (RESTCH mod. MM400). The particles were then left to dry at 105ºC to further analyse physicochemical parameters. The physicochemical parameters in the vermicompost samples were analysed as follows: electrical conductivity (EC) and pH were measured in a 1:10 water extract (w/v); moisture content was determined after drying to a constant weight at 105ºC for 24 h; total organic matter (TOM) content was measured by loss on ignition at 430ºC for 24 h; and total organic carbon (TOC) and total nitrogen (TN) were determined by burning the samples at 1020ºC in an automatic elemental micro-analyser (EuroVector Elemental Analyzer, Milano, Italy). After digestion (HNO_3/H_2O) (1:1, v/v) of dry samples in the microwave system (CEM, mod. MARS ONE), macronutrients such as P and K, among others (Ca, Cu, Mg, Fe, Mn, Zn), and toxic heavy metals (Cr, Ni, Cd, Hg, Pb) were measured by ICP-OES.

The humic-like content was measured in an extract with 0.1 M NaOH, from which fulvic acid-like C (C_{FA}) was separated through acid precipitation of the humic acid-like C (C_{HA}). The extracted (C_{EXT}) and supernatant (C_{FA}) were analysed in an automatic carbon analyser for liquid samples (TOC-V CSN Analyzer, Shimadzu Company, Kyoto, Japan). The water-soluble carbon (WSC) was measured in a 1:20 water extract (w/v) using the same automatic analyser for liquid samples.

Vermicompost and Biofilm Enzymatic Activity

The vermicompost samples were homogenized by grinding the aggregates in a ceramic mortar and adding H_2O. The water suspension was at a ratio of 1:50 (w/v), namely, 1 g to 50 mL H_2O. Suspensions were carried out at the moment of preparation or maintained at 4–5ºC for a maximum of 3 days. The biofilm was carefully separated from the substrate and scraped with a spatula until the plastic was clean. Later, the sample was homogenized at a ratio of 1:10 (w/v), namely, 0.1 g to 10 mL H_2O.

Carboxylesterase activity (CbE) (EC 3.1.1.1.) was measured by pouring aliquots (100 µL) from the sample and adding 380 µL of Tris-HCl 0.1 M buffer (pH 7.0). The enzymatic reaction was initiated by adding 20 µL of 1-naphthyl butyrate substrate (1-NB) (2 mM, final concentration) and waiting 5 min before stopping the reaction. The formed product (1-naphthol) was revealed by adding 50 µL of Fast Red ITR salt to 0.1% (w/v), dissolved at 2.5% (w/v), and Triton X-100 at 2.5% (v/v). Finally, the absorbance of the naphthol-Fast ITR complex was measured at 450 nm using an Asys HiTech UVM340 microplate reader (Asys HiTech Gmbh, Eugendorf, Austria). Carboxylesterase activity was expressed as nmol h^{-1} g^{-1} of dried substrate, determined by a calibration curve built for

1-naphthol. Control (without substrate) and blank samples (without vermicompost) were used to correct the background absorbance and non-enzymatic hydrolysis of the substrates, respectively.

Dehydrogenase (DHE) activity was measured by weighing 0.1 g of sample and adding 750 µL of Tris-HCl 0.1 M buffer (pH 7.0) + 1 mL INT. This was homogenised in a vortex and kept at 40ºC in a water bath for 1 hour in darkness (samples were shaken every 20 minutes). The reaction was stopped by adding 2.5 mL of stop solution prepared as a mixture of N-N0 dimethyl and ethanol in a 1:1 (v:v) ratio. Two random controls were prepared with 750 µL TRIS without INT. The plate spectrometer measurements were read at 450 nm.

To determine the catalase (CAT) (EC 1.11.1.6.) activity, 1 mL was collected from the 1:50 (w/v) aqueous suspension and dispensed with 125 µL of hydrogen peroxide (H_2O_2). It was put in a rotor for 10 minutes to allow the reaction to take place and then stopped with 125µL 3 M sulfuric acid.

Eisenia fetida Survival and Body Weight

After 7, 21, 30, and 45 days of the exposure assay, the earthworms were gently extracted from the feedstock of each replicate (Petri dish) by hand. Then they were counted for survival, weighed on a precision scale, and this information was recorded. At the end of the microcosm bioassay (45 d), the earthworms in each replicate were sampled.

Earthworm Biomarkers

Six earthworms randomly selected from each test replicate were used for this analysis; the selected earthworms were previously depurated (24 h) to eliminate the organic substrate of the gut tract. The earthworm's bodies were homogenised in ice-cold buffer (pH = 7.4) made of 25 mM sucrose, 20 mM Tris-HCl buffer, and 1 mM EDTA by milling with a potter (Heidolph Company). The homogenates were centrifuged at 9000× g for 20 min at 4ºC to obtain the post-mitochondrial fraction, which was aliquoted and stored at −80ºC until analysis.

The total protein content of *E. fetida* was determined in a 1:10 (v/v) aqueous dilution with bicichoninic acid (BCA). The reagent was heated at 60ºC for 15 minutes, and then read on the spectrometer at 630 nm. Acetylcholinesterase (EC 3.1.1.7) activity was

spectrophotometrically determined in the presence of 3 mM acetylthiocholine iodide as substrate and 0.1 mM DTNB (5.50-dithiobis-2-dinitrobenzoic acid) by measuring the increased absorbance during the kinetic reaction, read at 412 nm. The enzymatic reaction rate was quantified against a blank without substrate for each measurement. To subtract the spontaneous hydrolysis of the substrate, a second blank was performed without the sample. Acetylcholinesterase was expressed as nmol min^{-1} mg^{-1} protein.

To determine CbE, 100 µL of homogenized tissue was added to 380 µL 0.1 M Tris-HCl buffer (pH = 8.4) and 40 µL 1-naphthyl butyrate (1-NB) 20 mM. The tubes were incubated at 20ºC for 10 min and then centrifuged for 5 min at 10,000 rpm. Then 150 µL of supernatant was transferred to new microplates, and the formation of 1-naphthol was revealed after adding 50 µL of a solution containing 0.1% Fast Red ITR. The microplates were stored in darkness for 20 min, and then the absorbance of the naphthol–Fast Red ITR complex was read at 450 nm. Lipid peroxidation was measured in 50 µL of homogenized tissue added to 450 µL of reactive acid 2-thiobarbituric (TBAR) and butylhydroxytoluene (BHT). The reaction was maintained for 30 minutes at 90ºC. After that, 250 µL was dispensed in 96 deep-well microplates and read at 492 nm in a spectrometer. Enzyme activity was expressed as µg MDA / mg protein. Three randomized samples were carried out without TBAR.

The glutathione-dependent antioxidant enzymes glutathione reductase (GR) (EC 1.6.4.2) and glutathione S-transferase (GST) were measured using the method described by (Macci et al., 2009; Sánchez-Hernández, 2011), respectively. GR activity was determined in an aliquot of 50 µL of homogenized *E. fetida* body tissue in a reaction medium of 100 mM Na-phosphate buffer adjusted to pH 7.5, 1 mM oxidized glutathione (GSSG), and 60 µM NADPH. The kinetic reaction was measured at 340 nm in the spectrophotometer to determine the rate of NADPH oxidation. Specific enzyme activity was calculated using the extinction coefficient of 6.22 M^{-1} cm^{-1}. Glutathione S-transferase was measured in a reaction mixture containing 100 mM Naphosphate buffer adjusted to pH 6.5, 2 mM CDNB (1-chloro-2,4-dinitrobenzene), 5 mM reduced glutathione (GSH), and 30 µL of sample. The extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to express the specific enzyme activity.

3.5.4. Statistical Analysis

Paper 2: The IBM SPSS Statics V.28 software package was used for the statistical analyses. To assess the significant differences in the results measuring survival, weight

variation, exoenzyme activity in the vermicompost, and biomarkers, the multivariate general linear model (GLM) was used, considering the effect of the main variables (*E. fetida* presence, plastic format, APW presence). LSD tests were also conducted with Tukey-b and DMS as post hoc tests. We used factorial analysis of variance to determine the statistically significant differences between EF presence, type of APW, and the interaction between these two factors. When the differences were significant, one-way analysis of variance (ANOVA) and the least significant difference (LSD) were conducted to establish the significant differences between means. Normal distribution and variance homogeneity were checked using the Shapiro–Wilk and Levene tests, respectively, before ANOVA.

Paper 3: The ANOVA analysis was conducted to study the effects of the different treatments in the environments with and without earthworms. In addition, the multivariate general linear model (GLM) was used to assess the effects of the five variables considered (*E. fetida* presence, APW type, APW presence, APW size, and pre-treatment). In both statistical analyses, the Tukey-b was used as a post-hoc test. The statistical analyses were conducted using the IBM SPSS Statics V.28 software package.

3.6. Selection and building of microbial consortia (EXO-PMC) in vermicomposting processes polluted by AWP

Microorganisms were selected from the scientific literature with the potential capacity to biodegrade plastics. Individual and consortium cultures (in pairs or trios) of the selected candidates were combined and tested for their ability to grow from LLDPE as the sole carbon source. Log-phase suspensions (10 µl) of each strain were inoculated in sterile saline (0.9% NaCl, w/v) into 48-well microplates containing 1 ml of minimal salt medium added from virgin LLDPE powder at 1% (w/v) (<1 mm). The initial cell concentration of each strain was 10^7 cells/ml. The growth of consortium components was quantified after 15 and 30 days of incubation at 30°C and shaking at 110 rpm by plate counting on Nutrient Agar. Triplicates were included for each condition.

All individual cultures and strain combinations, except S. warneri JW24, showed a similar growth trend (Figure 19). Growth increased slightly in the range of 0.5 to 1 log units during the first 15 days and then decreased to values 1 to 2 log units below the initial cell concentration (7 log units). The cell viability of the *S. warneri JW24* monoculture decreased steadily.

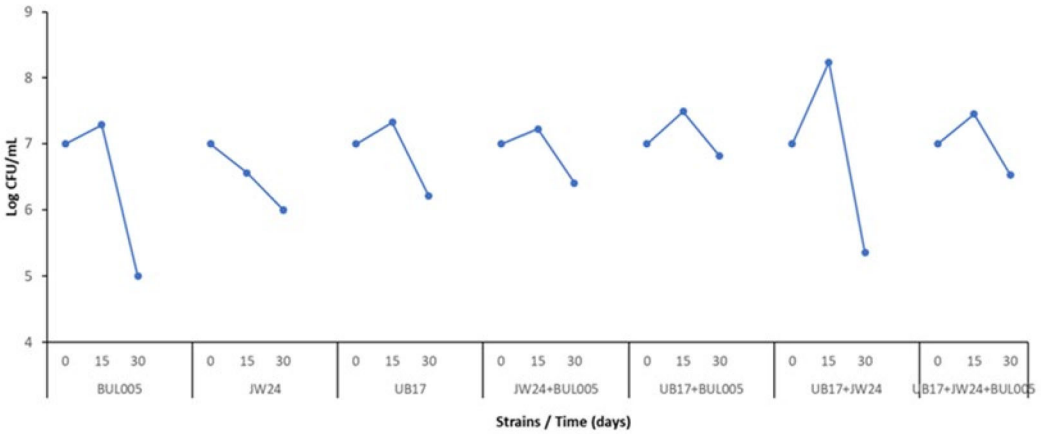


Figure 19. Growth curves of BUL005, JW4, and UB17 in monoculture and mixed cultures (pairwise or trio) on LLDPE at the sole carbon source.

Consequently, binary consortia comprising *B. altitudinis UB17* and *P. stutzeri BUL005* or *S. warneri JW24* are identified as promising species for LLDPE degradation. A potentially synergistic interaction is expected according to the improvement in growth levels observed when combined. However, the consortia appear stable over a certain period; competitive interactions or other factors such as carbon depletion or available metabolites could be responsible for the loss of viability after 30 days of incubation.

Table 13. Microbial consortia selected for further activities

	Name	Composition	AWP-Degrading capabilities
EXO-PMC	Consortium Exo: Bs-Pp (C4)	*Bacillus subtilis* RBM2 *Pseudomonas putida* REBP7	Use LLDPE, LDPE, PET and PS (recycled and virgin) as the sole carbon source. Cause weight loss of LDPE-recycled (11%), LLDPE-virgin (13%) after 30 days

Comprehensive analysis of all tested consortia revealed that two microbial consortia are suitable for application as Exo-AWP degraders in compost or outside the body of earthworms due to their ability to grow from all types of AWP and reduce the weight of LLDPE and PET plastic (Table 13).

3.7. Extraction and production of beneficial microorganisms (ENDO-PMC) in *E. fetida* as prebiotics present in vermicomposting processes polluted by AWP

Iidentification and isolation of microbiome were carried out in research group BIO-175 at Univ. Almeria. Microorganisms isolated from the gut of earthworms exposed to plastic in a vermicomposting process (from the previous trials) were selected to be applied as endogenous beneficial microorganisms with probiotics characteristics (Table 14):

4. **Table 14.** Consortia used with *E. fetida*

	Name	Composition
ENDO-PMC	Consortium Ef: Bl-Pp-Bs	*Bacillus licheniformis S-ALME2-B2* *Pseudomonas putida ALME2-B2* *Bacillus sonorensis ALME2B3*

To isolate endogenous plastic microbial consortia (ENDO-PMC) from the digestive tract of *E. fetida*, specimens were surface sterilized (to prevent cross-contamination with epithelial microbiota) by sequential immersion in sterilizing solutions. The sterilized specimens were then placed in Eppendorf tubes and homogenized. Subsequently, 10-fold serial dilutions were performed, and 100 µL aliquots were plated on both APHA and Rose Bengal (RB) (Panreac) culture media for the enumeration of general bacteria and fungi, respectively. Petri plates were incubated for 24 hours for bacteria and 72 hours for fungi at 30 °C. Colonies that stood out as the predominant ones in the test samples were chosen for utilization as probiotics in the following assay. Strains exhibiting easy culturing conditions, abundance in the overall population, and non-pathogenicity were selected, isolated and taxonomically identified. In order to taxonomically identify the selected strains, pure cultures of each strain were subjected to genomic DNA extraction utilizing a heat shock procedure: (5 min /96 ºC and 5 min /4 ºC). Molecular identification through 16S rRNA gene amplification was executed utilizing universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907R (5′-GGTTACCTTGTTACGACTT-3′) (Isik et al., 2014). The PCR thermal conditions included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 30 seconds, and a single final step of 72 °C for 10 minutes. Amplicon size was verified through electrophoresis in 1% agarose gel stained with GelRed (Biotium Inc., Hayward, CA, United States). Finally, the amplicons purification was performed using E.Z.N.A cycle pure kit and sequencing was performed through the dideoxynucleotide cycle sequencing method on an ABI 3500 genetic analyzer.

3.8. Production of stable beneficial microorganisms (PMC-consortia)

The production of microorganisms was replicated in culture media on a 10L scale. Culture conditions were adjusted according to information developed for high-cell density fermentation of *Bacillus sp.*, *Pseudomonas sp.*, and other bacterial species.

Subsequently, the physical-chemical parameters of the fermentation process were optimized. Additionally, the carbon and nitrogen sources of the fermentation media were optimized to establish fed-batch processes. The growth rate and maintenance of the AWP degrading activity were verified, and the cultivation time was selected according to the production peaks.

Development of fermentation processes (for all *Bacillus sp.* Species, the same basic medium was used): yeast extract 10.0 g/L, meat extract 3.0 g/L, NH_4Cl 2 g/L, $MgSO_4$ 0.2 g/L and KH_2PO_4 2.0 g/ L.

After an initial growth phase of 4 to 6 hours, feeding consisting of glucose and yeast extract was started using a feeding dose adapted to the respective species. Once the fermentation was completed, the biomass was separated using a batch centrifuge and stored under different conditions. Finally, the viable cell count was determined.

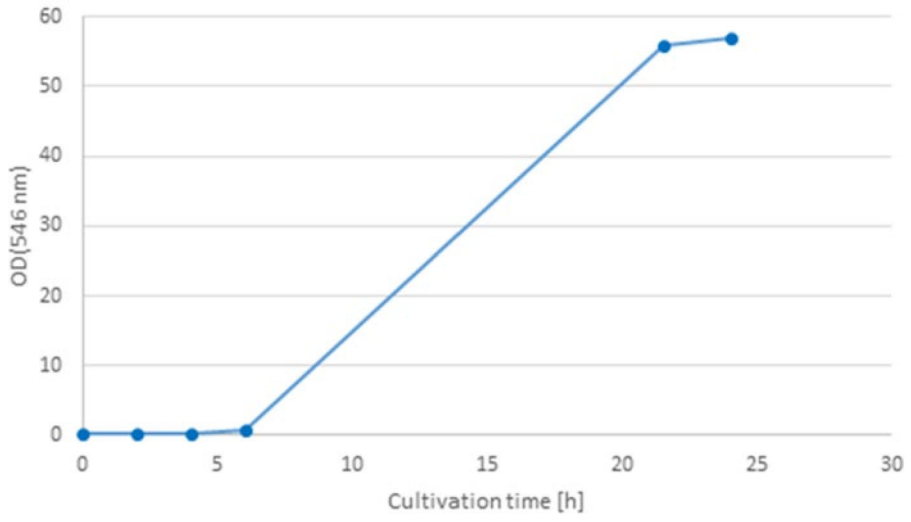


Figure 20. Fed batch fermentation of B. subtilis J2 - growth diagram

All the bacterial species investigated showed good growth and reached a high cell density. For all bacteria, the maximum number of cells was reached at most after 24

hours of fermentation. The maximum cell density was not reached after 24 h of fermentation; however, after introducing an additional sampling point at 20 h, it became evident that the maximum cell mass was reached at 20 h. As an example, Figure 20 shows the growth diagram of *B. subtilis J2.*

The best bacterial biomass yield values were found in *Bacillus sp.,* which were more than 40 g per litter of fermentation broth. For the fungus *Fusarium sp*., a biomass yield of >150 g per litter could be achieved. The highest cell counts achieved were approximately 3 x 10^9 cfu per ml for *Bacillus sp.*

3.8.1. Development of suitable storage conditions for microorganisms and preparation of stable PMC formulations

To find optimal storage conditions for isolated cultures of microorganisms, it was decided to opt for freeze-drying. The proportion of the collected cells was suspended at 16.7% w/w in an aqueous solution containing 0.9% NaCl and 20% w/w lactose and lyophilized. At regular intervals the cell count was detected.

All freeze-dried samples showed viable counts of 50% and below. Evidently, the freeze-drying process causes a substantial loss of living cells. However, for long-term storage, freeze-dried ones will have advantages. Furthermore, in the trials it was considered that the freeze-dried ones were easier to handle.

Microbial formulation is a vehicle-based preparation to provide microbes with better survival for a longer duration. The stability of the microbial inoculants was used in the following formulations: Powdery formulations for all consortia (ENDO- and EXO-PMC).

For the preparation of powder formulations, the viability of all strains was determined by lyophilization using skim milk (10% w/w) as cryoprotectant. Cell suspensions in protective medium (3 ml) were placed in serum vials and frozen at −80°C overnight. The vials were then placed in a lyophilizing chamber (Telstar, LYOQUEST-55) at −45 °C and 0.05 mbar for 12 h. After lyophilization, the vials were sealed and stored at +4°C. Viable counts were determined before and after lyophilization, and viability was defined as the percentage ratio of viable cells after lyophilization to viable cells before lyophilization. All strains showed high levels of viability (Table 15).

Table 15. Stability of microbial strains to freeze-drying (lyophilization)

Microorganism	Viability (%)	Stability upon freeze-drying
Bacillus licheniformis S-ALME2-B2	85	Stable
Bacillus sonorensis Alme2B3	90	Stable
Bacillus subtilis RBM2	88	Stable
Pseudomonas putida ALME 2B2	89	Stable
Pseudomonas putida REBP7	84	Stable

Consequently, formulations from the different consortia can be obtained by mixing equal amounts of cellular biomass from each microbial component before adding skimmed milk and freezing. Each initial cell suspension must be adjusted to provide the final cell concentration required in the formulation, the recommended levels of which are 10^9-10^8 CFU/g for bacteria and 10^7-10^8 spores/g for fungi.

3.9. Design and development of a vermicomposting bioassay for exposure to the presence of AWP with beneficial microorganisms

3.9.1. Experimental design

This laboratory-scale bioassay was designed to reproduce the conditions of the vermicomposting process of agro-industrial organic waste in the presence of AWP and an inoculation of three different microbial beneficial consortia.

In this section, we proceed to describe the types of consortia used. ENDO-PMC consortia was identified and isolated from the gut of *E. fetida* exposed to AWP presence. The identified bacteria belonged to two genera, *Bacillus sp.* and *Pseudomonas sp.* It has been tested due to a possible probiotic effect on earthworms, which may lead to the production of fortified *earthworms* with APW degrader capacities. EXO-PMC is based on bacteria (*Bacillus sp.* and *Pseudomonas sp.*) and fungi (*Alternaria sp.*) previously selected for their AWP-degrading capabilities. MIXc (ENDO+EXO-PMC) both consortiums were tested in the same inoculated treatment to discriminate the possible synergetic effect in produce *E. fetida* with fortified capacities.

3.9.2. Experimental set-up

The bioassay consisted of an incubation of 100 adult of *E. fetida* individuals for 360 hours in plastic containers (4L) using 640 g f.w. of feedstock adjusted with distilled water to 70% moisture content. Then, 4g of AWP material was added per replicate (1.25 % f.w. proportion) (Sáez et al., 2022). The incubation containers were kept in isolated chambers under controlled conditions (20° ± 2 °C and darkness). After 0, 72, 144, 216, 288, and 360 hours of exposure, survival and body weight variation were determined. For this, earthworms were carefully extracted from the Plastic box (4L) of each replicate by hand sorting, counted for survival, weighted, and recorded to obtain the mean body weight of each treatment. During the exposure time of the bioassay, when mortality was observed, the earthworms were immediately removed from the plastic container. In addition, at the end of the bioassay, the final materials obtained were treated in the same way as the previous bioassay, with sampling at 0 and 360 hours.

We used two different AWP materials (LDPE and MIX-AWP) at microplastic size and the presence of a three-microorganism consortium was used. MIX-AWP consists of the following proportions of plastics: 32.5% LDPE + 32.5% LLDPE + 20% PS + 15% PET. The earthworms and substrate used were the same as in the previous bioassay. Considering the main factors established in the experiment (plastic type, pre-treatment or not, *E. fetida* presence, and AWP presence), six different types of experimental devices with three replicates each were prepared: a) feedstock + AWP - earthworms + PMC; b) feedstock + earthworms - AWP presence + PMC; c) feedstock + AWP + earthworms + PMC; d) feedstock + AWP - earthworms - PMC; e) feedstock + earthworms - AWP presence - PMC; f) feedstock + AWP + earthworms - PMC.

In Figure 21 we can see a graphic diagram of the bioassays carried out and the parameters analysed.

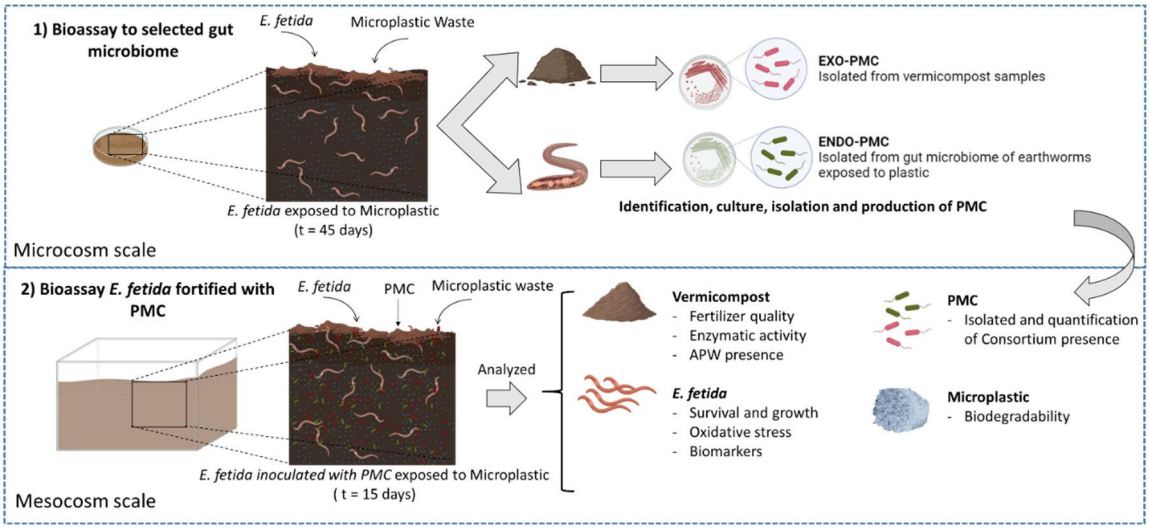


Figure 21. Graphic diagram of the bioassays carried out.

4.2.3. Analytical methods

The same analytical methods were used as described in Section 3.5 “Design and development of vermicomposting bioassays for exposure to the presence of AWP”.

4.2.4. Statistical Analysis

The ANOVA analysis was conducted to study the effects of the different treatments in environments with and without earthworms, with and without PMC consortiums, and in the presence and absence of AWP. In addition, the multivariate general linear model (GLM) was used to assess the effects of the five variables considered: *E. fetida* presence, type of AWP, AWP presence, type of PMC-consortia and PMC-consortia presence. In both statistical analyses, the Tukey-b was used as post-hoc test. The statistical analysis was conducted using the IBM SPSS Statics V.28 software package.

4. Results and Discussion

4. RESULTS AND DISCUSSION

4.1. Representative collection and characterization of main plastic material used in Agrifood sector

For this thesis, a representative catalogue of fossil plastics, picked individually from agriculture sector was developed (Table 16). This selection has been confronted with the literature data revision and the objectives of RECOVER project. All these materials were used to carried out all the experiments linked to the different publications, including also blends between LLDPE and LDPE, and different use status and film or particle format.

Table 16. Main characteristics of plastic samples used in PhD.

AWP type	Origin	Used	Film Size/ Thickness	Nº Paper	Function
LDPE	Solplant	Virgin/ Used	1cm^2/ 20 µm	1,3,4	Crop protection
LLDPE	Repsol	Virgin/ Used	1cm^2/ 50 µm	1,3,4	UV protection
PET	UNIPI	Virgin/ Used	1cm^2/ 200 µm	1,3,4	Plastic covers and mulching films
PS	UNIPI	Virgin/ Used	1cm^2/ 200 µm	1,3,4	Carrier bags
LLDPE + LDPE	Solplant	Virgin/ Used	1cm^2/ 50 µm	1,2,3	Covers in greenhouses
EPS	UNIPI	Virgin/ Used	1cm^2/ 1280 µm	2	Irrigation pipes
PP	ITENE	Virgin	1cm^2	1	Nets for collecting
MIX	UMH	Virgin	MIX µm	4	Geomembranes

AWP type	Number of MP items / 100 mg	% Fraction size of MP in weight (mm)				
		>2	**1-2**	**0.2-1**	**0.05-0.2**	**<0.02**
LDPE	137±16	7.5%	78.7%	16.2%	0.3%	0.2%
LLDPE	352 ±43	25.7%	61.1%	14.4%	0.3%	0.6%
PET	247±28	30.6%	53.2%	14.0%	2.4%	1.9%
PS	284±32	6.4%	73.4%	17.5%	5.0%	1.1%
LLDPE + LDPE	248±18	18.8%	68.5%	13.0%	0.3%	0.5%
EPS	-	-	-	-	-	-
PP	-	-	-	-	-	-
MIX	-	-	-	-	-	-

*MIX: 32.5% LLDPE + 32.5 LDPE + 20%PET + 15% PS.

4.2. Effects of different pre-treatment approach used on AWP

In this PhD we have applied an approach to produce degradation of agricultural plastics (AWP) through different pre-treatments (Table 17). We employed thermal treatments, photo-oxidation treatments (Table 18), chemical/thermochemical treatment and E-beam treatment (Table 19).

Table 17. Main characteristics of plastic samples used in pre-treatments.

Plastic type	Format	UV	e-Beam	Chemical	Thermochemical
LDPE	Film	D	NS	D	D
	MP	D	D	NS	NS
LLDPE	Film	D	D	D	D
	MP	D	D	NS	NS
PET	Film	D	D	D	D
	MP	D	D	NS	NS
PS	Film	D	D	D	D
	MP	D	D	NS	NS
LLDPE + LDPE	Film	D	D	D	D
	MP	D	D	NS	NS
LLDPE + LDPE BF	Film	D	D	NS	NS
	MP	NS	NS	NS	NS
LLDPE + LDPE PF	Film	D	D	D	D
	MP	D	D	NS	NS
PP	Film	D	NS	D	D

* BF: Black film; PF: Perforated film; D: done; NS: non-selected.

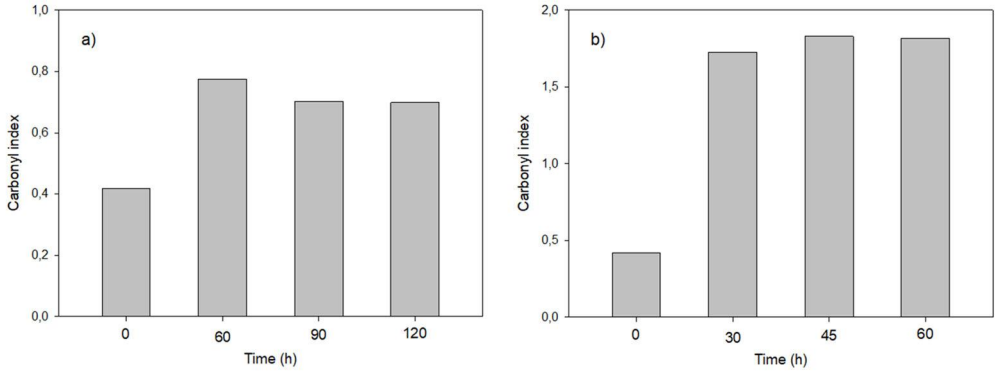
Table 18. Details of the UV chamber characteristics.

Type UV	W x chamber	Irradiated surface (m^2)	Power consumption (kW/m^2 h)
B	20	0.2479	0.161
C	16.7	0.2479	0.135

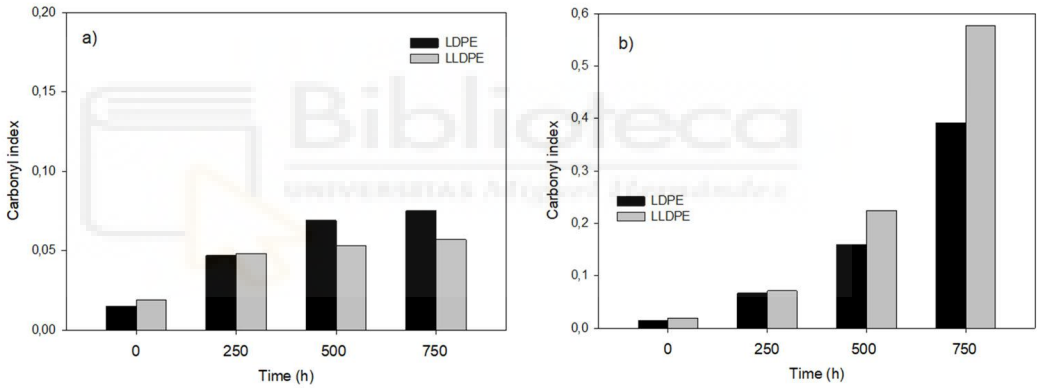
Table 19. Values of FTIR carbonyl index used in the representative polymers after applying the e-beam pre-treatments.

E-beam pre-treatment	Carbonyl index			
	PS	LDPE	LLDPE	PET
0 kGy	0.309	0.039	0.070	0.999
100 kGy	0.277	0.063	0.087	1.002
200 kGy	0.331	0.065	0.094	1.002
500 kGy	0.373	0.097	0.125	1.004

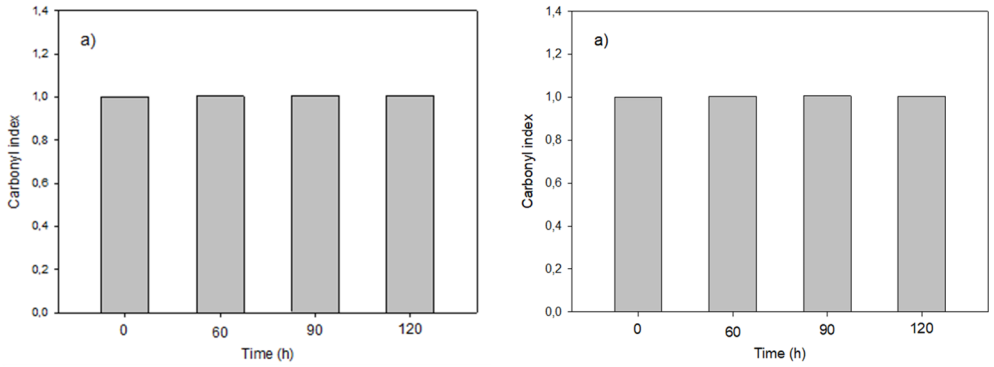
In order to estimate the effect of each pre-treatment on the degradation of the AWP, we used several techniques: visual parameters, FTIR infrared spectrophotometry and Thermal gravimetric analysis (TGA) (Details can be found in the section 3.3.). Figure 22 summarizes an example of the developed treatments by UV in the different AWP:



representative PS sample



representative LDPE and LLDPE samples



representative PET sample

Figure 22. Values of FTIR carbonyl index used in the representative AWP types after applying during different exposure times the photo-oxidation pre-treatments: a) UV-B; b) UV-C.

The most important results of the pre-treatments by type of plastic are described below:

Regarding PS, our results indicated that after photo-oxidation and e-beam assays, the highest impact on surface degradation was associated to the use of UV-C radiation with a significant increase in all the indexes considered after 45h. UV-B effects were, in general, least than a half compared to UV-C. Significant variation of UV-C was noticed at 30h. E-Beam effect was poor in terms of increment of indexes compared to non-treated plastic probes. In general, no linear response of photo-degradation with time of exposition was stablished. However, when we consider thermogravimetric parameters (TGA and DTG based on loss of weight and energy consumption during combustion), we can assess the impact of pre-treatments on the whole plastic probe, not only surface effects. For PS, UV-B at 120h of exposition produced the highest significant decrease in starting degrading temperature (T98% and T95%), more than 80 and 60ºC and lower compared to untreated. UV-C at 30h was the second-best pre-treatment using TGA, without correlation between time of exposition and degradation, the opposite being true for UV-B. E-beam induced reduced effects on TGA compared to UV pre-treatments. Thermochemical pre-treatments achieved significant increases of the studied indexes on PS probes. The statistical analysis showed that the concentration of this chemical reagent was the factor that most influenced-on PS degradation, followed by the combination of the three factors (temperature, time and concentration). The pre-treatments with ammonium persulfate and chromic mixture showed the strongest oxidizing effect, even observing a disintegration of at least 50% of the plastic sample at the exposure temperature of 70 ºC, independently of the exposure time (6 or 12 days). Apart from this effect, considering separately the effect of each chemical reagent in the exposure conditions established, all the chemical reagents used caused the highest degradation effect with the greatest concentration (100%), as it can also be observed in the changes of the different functional groups of the FTIR spectra.

Concerning PET, our results revealed that UV-B was more effective than UV-C on this type of plastic, considering all the FTIR indexes, which is in concordance with the results observed by Fagerburg and Clauberg (2003). The exposition time was not correlated with the increase of indexes. E-beam radiation in the considered conditions did not induce a significant increase of FTIR indexes, with weak increases compared to non-treated samples and also not related with e-beam dose. For the pre-treatment with UV-B, the exposure time of 90 h was the most effective. Considering TGA and DTG assessment, PET seemed to be not affected by pre-treatments, with less than 2% of starting temperature reduction (T98%) in the most favourable scenario (e-beam) and

observing no differences for T95% and T50%. These results are quite different compared to PS that showed >25% reduction for T98%. In addition to this, no effect on time of exposition was observed for UV or e-beam pre-treatments. The degradation effect was mainly dependent on the chemical reagent used and on the concentration of this reagent. In general, the highest concentration (100%) seemed to produce the greatest polymer degradation. Disintegration of plastic probes was observed with all the reagents especially with concentrated AP in all the scenarios. The chromic mixture produced disintegration effects especially in long-term experiments. PET seemed to be the most chemically affected plastic type of the selected collection.

Regarding PE our results confirmed the refractory nature of PE plastics, LLDPE and LDPE, where significant increases in irradiation energy/time must be applied for UV and e-beam pre-treatments to obtain indexes variation, compared to PS and PET.

On LDPE probes, the results obtained after the photo-oxidation pre-treatments have shown the highest effect on surface oxidation with the UV-C pre-treatment at the highest exposure time (750 h), obtaining the greatest values for all the indexes considered, which indicates that this pre-treatment produced the highest degradation in the LDPE representative sample used. These results also indicate the high resistance of LDPE to photo-oxidation. Using TGA approach for stablishing modifications on plastic integrity, we observed 33ºC reduction of temperature for T98% conditions using UV-C, but e-beam was efficient as pre-treatment with a higher reduction than UV-B (20 vs 6ºC), also being the pre-treatment that induced the highest reduction at T95% conditions. Considering the thermochemical approach, all the three oxidants at the highest concentration (100%) produced the greatest degradation, including also disintegration of the sample, with chromic mixture (CM) and aqua regia (AR). In particular, aqua regia was especially efficient in oxidizing LDPE. If we consider a soft approach (no disintegrating purposes), the lowest concentration of reagent (33%) and at the exposure conditions of 70 ºC during 6 days with CM and AR constituted the optimal pre-treatments. Considering Pareto charts, the concentration of the chemical reagent was the most significant factor followed by the combination of time and temperature.

For LLDPE, also UV-C pre-treatment at the highest exposure time (750 h) was the most degradative pre-treatment, with similar behaviour in the different FTIR indexes tested than LDPE in both LLDPE probes (P16 and P1). UV-C pre-treatments were very effective in altering the plastic integrity, especially in the range of 500-750h of exposition, with decrease in T98% conditions ranged between 52-154ºC. E-beam irradiation seemed to

decrease in T98% conditions ranged between 52-154ºC. E-beam irradiation seemed to induce higher degradation effects compared to UV-B exposition. When we analysed the thermochemical pre-treatments effects on LLDPE, the highest concentration (100%) produced the greatest degradation as in LDPE, but the response to the exposure conditions was different. Disintegration was only observed for the chromic mixture and aqua regia pre-treatments. Non-linear response to concentration was observed if disintegration is not considered, with different effects depending on the chemical oxidant used. Pareto charts for LLDPE samples pre-treated with aqua regia indicated the most significant degradative effect of the factor temperature, on the contrary than in the results observed by the other reagents.

In general terms, the most effective treatment for each plastic type is shown in Table 20 (The full results can be found in Annex 7.1).

Table 20. Results of cost assessment for optimal pre-treatments and plastic types.

Plastic type	UV-B	UV-C	E-beam	Thermochemical
PS	60 h 2.5d 9.7kW	45h 1,9d 6.1kW	500 kGy <1d 3.6 kW	AM ≈ CM > AR Concentrated>diluted 12d>6d 35ºC ≥ 70ºC 8 kW
Cost-effective assessment	T-M / E-L / Env-L	T-M / E-L / Env-L	T-L / E-L / Env-L	T-H / E-L / Env-H
PET	90h 3.7d 14.5 kW	30h 1,3d 4.0 kW	100-200 kGy <1d 2.8 kW	AM ≈ CM > AR Concentrated>diluted 12d>6d 70ºC ≥ 35ºC 16kW
Cost-effective assessment	T-M / E-L / Env-L	T-M / E-L / Env-L	T-L / E-L / Env-L	T-H / E-M / Env-H
LDPE	500-750 h 20-31d 80-120 kW	750 h 31d 101 kW	1000 kGy <1d 4.9 kW	AR > CM > AM Concentrated>diluted 12d>6d 70ºC ≥ 35ºC 16kW
Cost-effective assessment	T-H / E-H / Env-L	T-H / E-H / Env-L	T-L / E-L / Env-L	T-H / E-M / Env-H
LLDPE	500-750 h 20-31d 80-120 kW	750 h 31d 101 kW	1000 kGy <1d 4.4 kW	CM ≈ AR > AM Concentrated>diluted 12d>6d 70ºC ≥ 35ºC 16 kW
Cost-effective assessment	T-H / E-H / Env-L	T-H / E-H / Env-L	T-L / E-L / Env-L	T-H / E-M / Env-H

AM: ammonium persulfate; AR: aqua regia; CM: chromic mixture; T: time consuming costs; E: energy consuming costs, Env: Environmental cost; L: low; M: medium and H: High.

Considering all pre-treatment types, the thermochemical assays with AM/CM/AR reagents seemed to be strongest approach, able to affect not only surface of the plastic probes but all the plastic material. De la Fuente et al. (2020) observed significant changes in carbonyl index for PE derived plastics, but without any disintegration using weaker reagents (citric acid, nitric acid). The AM/CM reagents produced higher effects than AR, being time and temperature cooperative factors for disintegration/degradation.

The effect of UV radiation was plastic-dependent, with more effective results with UV-C for PS, LDPE and LLDPE, and UV-B for PET. The suggested time of UV exposition to promote significant changes in plastics is also time-dependent, being in the range of 30-90h for PS and PET but 500-750h for PE (LDPE and LLDPE), due to its more refracting structure.

E-beam radiation did not show consistent increases on FTIR indexes, indicating no so significant surface oxidation than that obtained with UV. However, considering TGA assessment, loss of integrity was promoted by e-beam considering the decrease on the temperature to achieve T98 and T95% conditions, and therefore e-beam pre-treatments must be considered especially for PS and PE plastics, PET being the least affected on these integrity parameters. Thus, e-beam pre-treatment should be included into the selected operations to be tested for promoting-enhance biodegradation, even in the case of PE derived plastics, where the use of higher e-beam doses (up to 1000 Gky) did not show significant increases.

The UVC (250h) pre-treated film plastics were included as treatments in the vermicomposting bioassays of Paper 3 (section 8.3.). The UVC pretreated AWP exerted a significant effect on the physicochemical parameters of the vermicompost obtained, altering pH, EC and nutrient content (TOC, K and C_{HA}). This could be attributed to changes occurring at the physical and/or chemical level, inducing alterations in the polymeric structure of AWP materials, rendering them more readily available for biological attack (Shah et al., 2008; Urbanek et al., 2021). The application of UV pre-treatment to AWP only affected enzymatic activities were CbE and DHE, with no significant effect observed on CAT activity. The lower enzymatic activities observed (CbE and DHE) could be related to reduced biological oxidation activity of the total organic carbon (TOC) in the substrate due to the potential release of toxic compounds derived from plastic materials when physically and/or chemically modified. On the other hand, UV pre-treatment does not appear to have a significant effect on the studied biomarkers (CbE, AChE, and lipid peroxidation) in the tissue of *E. fetida.*

4.3. Effects of AWP presence on *Eisenia fetida*

We have developed different experiments to establish the level of earthworm damage in the presence of AWP as a xenobiotic that alters the vermicomposting process developed by the earthworms. In this section we will show the results of the effect on the earthworm for the following damage or alterations: survival, morphological effect, and response of biomarkers. The results have been published in the Paper 2 and 3 (7.2 y 7.3).

4.3.1. Survival

The results suggest that the presence of AWP had a significant negative impact on both the survival and body weight variation of *E. fetida*. The highest mortality was mainly observed at the beginning (7 days) of exposure assay. Plastics that resulted in the lowest survival values were LLDPE, the mixture of LDPE + LLDPE and LDPE + LLDPE-Black film (additive), causing up to 25% decrease compared to the control. In the PET, PS and LDPE treatment, statistical effect was detected, but with a decrease of the survival rate of less than 10% compared to the control treatment (Figure X a) and b)). The observed decline in survival rate for epigeic earthworms exposed to plastics under vermicomposting conditions has been described as a set of biotic factors affecting various physiological processes, such as respiration rate, reproduction rate, feeding rate, and burrowing activity (Yadav et al., 2011).

4.3.2. Morphological effect

Concerning the body weight variation of earthworms in the presence of AWP, *E. fetida* showed higher susceptibility to negative morphological effects when exposed to LLDPE and/or LDPE + LLDPE plastic treatments, resulting in similar values of negative body weight variation. Similar trends were observed for the other treatments (PET, PS and LDPE), with minor differences compared to the control treatment (Figure X c) and d)). The substrate corresponding to the control treatment maintained a higher density of earthworms with lower mortality, and an improvement in the body weight of *E. fetida* was also evident compared to the substrate with plastic. This indicates that the nutritive capacity of the substrate material was not a limiting factor for the development of *E. fetida* in the experimental bioassay. Thus, these results may suggest that body weight loss in the plastic treatments could be induced by stress in the physiological activity of the earthworms.

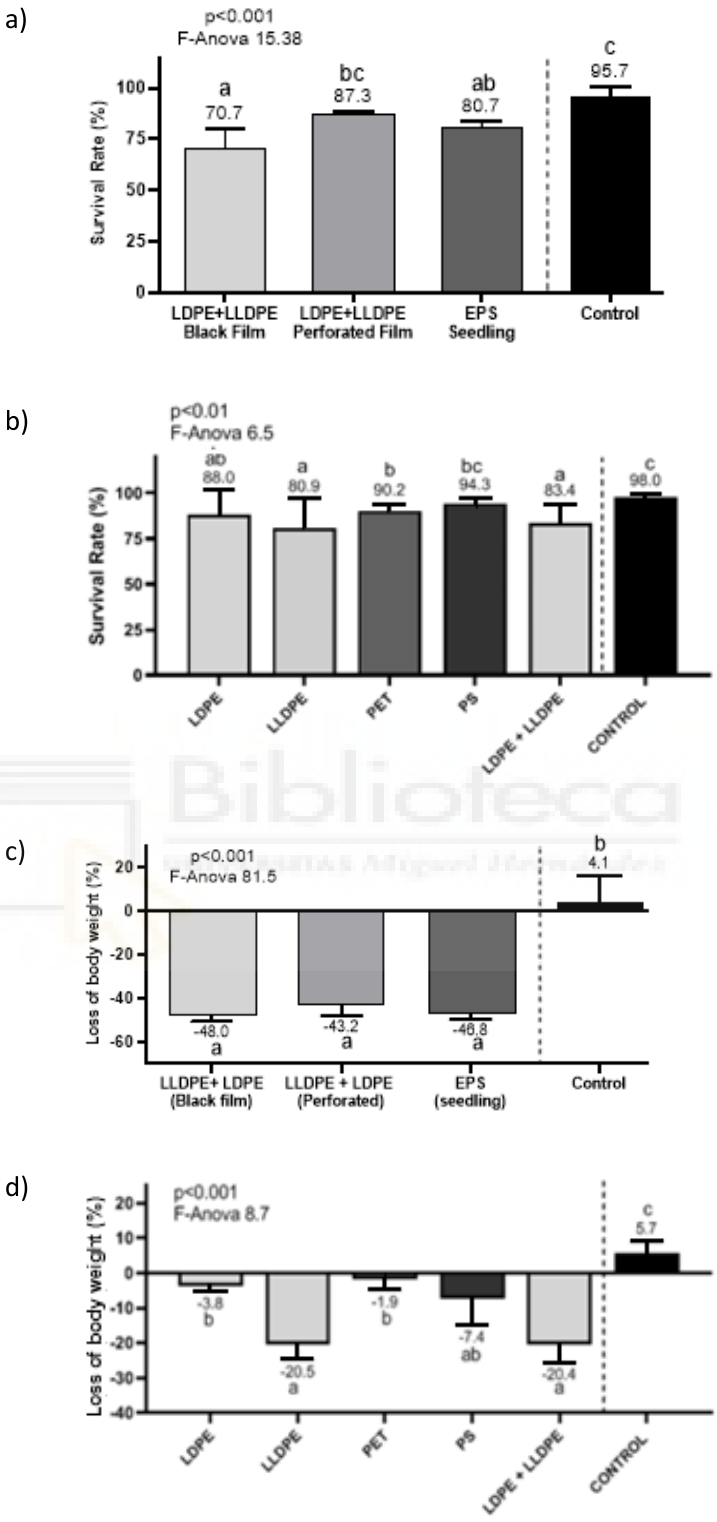


Figure 23. *Eisenia foetida* survival and morphological effect: a) survival rate (Paper 2), b) survival rate (Paper 3); c) morphological effect (Paper 2); d) morphological effect (Paper 3)

4.3.3. Response of biomarkers

In this section, the response of *E. fetida* to AWP by quantifying distinct biomarkers is described: carboxylesterase (CbE), acetylcholinesterase (AChE), lipid peroxidation, GST activity and Glutathione reductase.

Our study suggests that the treatments with the AWPs including LLDPE in the backbone polymer formation (LLPDE, LLDPE + LDPE, LLDPE + LDPE Black film and Perforated film) and EPS were the only treatments that showed the mean value of CbE activity close to the control treatment. On the other hand, the highest CbE activity mean was observed in the treatments where *E. fetida* was exposed to PET and PS, and to a lesser extent in LDPE. The results obtained seem to suggest that different types of AWP activated different molecular mechanisms in *E. fetida.* The chemical nature of the plastic polymer will be determinant in the pathaway followed by CbE. Chen et al. (2020) also investigated the effect of different types of microplastics and reported a variable mode of action depending on the type of plastic, as well as the shape, size, and possible influence of additives.

In terms of Lipid peroxidation activity, the results obtained are contrary to those expected, since we observed a decrease in lipid peroxidation when *E. fetida* was expose to LLDPE + LDPE (Black film), LLDPE + LDPE (Perforated film) and EPS (Figure Xc). On the other hand, when *E. fetida* was expose to LDPE, LLDPE, PET and PS, we do observe a significant increase in peroxidation activity (Figure Xd)). The presence of LDPE and PET induced the highest values. The increase of this kind of enzyme activity, as in other studies (Sarker et al., 2020; Wang et al., 2020), has been shown to be due to the fact that MP (with a variety of chemical composition) can lead to skin damage, tissue lacerations, altered immunity, and neurotoxicity in terrestrial organisms (such as ciliates, springtails, and earthworms). Other studies have even revealed that exposure to MP (like HDPE, PP and LDPE) with a size of less than 300 μm by *E. fetida* (Chen et al., 2020; Jiang et al., 2020) caused the development of clear inflammatory processes between the intestinal epithelium and chloragogenic tissue, sometimes leading to fibrosis and congestion (Rodríguez-Seijo., 2018).

AChE serves as a biomarker of neurotoxicity, breaking down acetylcholine to mitigate the neurotoxic effects of contaminants in various species (Zhang et al., 2020). Like CbE, the presence of AWP materials resulted in an AChE interaction. AChE activity in *E. fetida* exposed to LDPE, LLDPE, PET, PS increased significantly compared to the control, with the highest levels observed in the presence of PET and PS (Figure Xf)). In LLDPE +

exposed to LDPE, LLDPE, PET, PS increased significantly compared to the control, with the highest levels observed in the presence of PET and PS (Figure Xf)). In LLDPE + LDPE (Black film), LLDPE + LDPE (Perforated film) and EPS, there are not no significant changes compared to the control (Figure Xe). Previous studies have also indicated an increase in AChE activity when *E. fetida* is exposed to plastic materials, suggesting that the presence of AWP stimulates the neurotoxicity response in *E. fetida* by exerting a specific regulatory effect on neurotoxins (Chen et al., 2020; Zhong et al., 2021; Zhang et al., 2020). For instance, Chen et al. (2020) observed increased AChE activity in *E. fetida* exposed for 21 days and 28 days to 1.0-1.5 g/kg LDPE in soil. Zhong et al. (2021) also reported increases in AChE activity in a study on the effect of MP in sludge in the vermicomposting process.

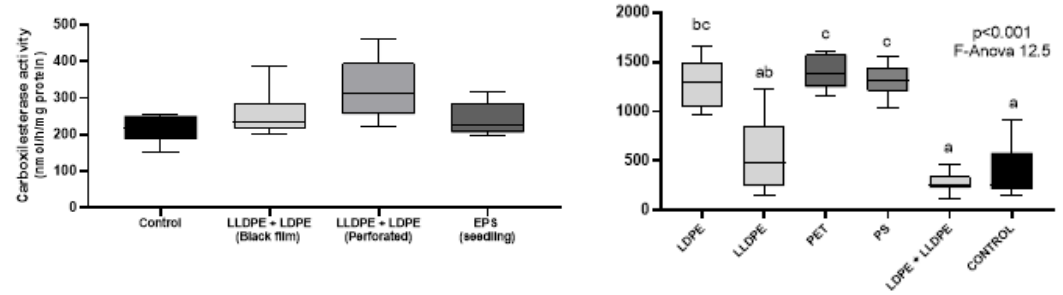
In addition, only for LLDPE + LDPE (Black film), LLDPE + LDPE (Perforated film) and EPS, glutathione S-transferase (GST) activity and Glutathione reductase was measured for determined the effect of plastic on antioxidant defences. In GST activity, the response was a significant increase in earthworm body tissue activity after exposure to all the plastic material tested. The results of the glutathione reductase showed a significant increase in *E. fetida* exposed to LLDPE + LDPE (Black and Perforated film) (Paper 2, Annex 7.2.).

A summary of the most significant effects on EF biomarkers are shown in table 21 and Figure 24.

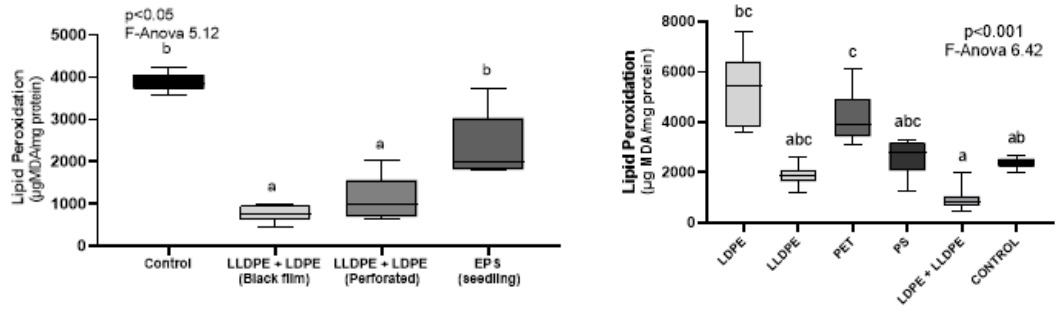
Table 21. Significant effect produced by the presence of AWP on *E.* Fetida: survival, body weight and response of biomarkers.

Significant effect			**Featured AWPs**
Morphological response	Survival (%)	↓	All of AWP tested
	Body weight (%)	↓	All of AWP tested
Biomarkers response	CbE ($\mu mol\ h^{-1}\ mg^{-1}$ protein)	↑	LDPE, LLDPE, PET & PS
	AChE ($nmol\ min^{-1}\ mg^{-1}$ protein)	↑	LDPE, LLDPE, PET & PS
	Lipid peroxidation ($\mu gMDA\ mg^{-1}$ protein)	↑	LDPE, LLDPE, PET & PS

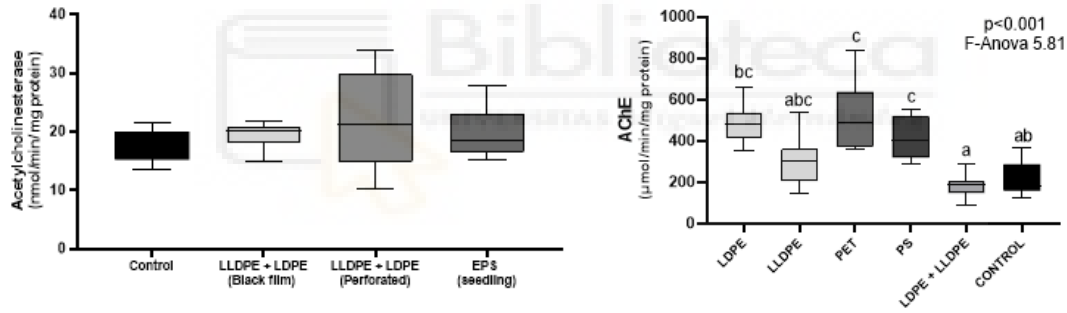
*↓: Decrease in value, ↑: Increase in value and =: No change in value.



CbE activity



Lipid peroxidation activity



AChE activity

Figure 24. CbE, Lipid peroxidation, AChE activity in *E. fetida* body tissue affected by the type of AWP.

4.4. Effects of the presence of AWP in vermicompost obtained

In this section, the results of the effect of the presence of AWP on the vermicompost obtained are described. The results have been published in the Paper 2 and 3 (Annex 7.2. and 7.3.).

4.4.1. Agronomic quality

The pH values at the end of the bioassay (45 d) remained within a suitable range for earthworm and microorganism activity (5.5–8.5) (Yadav et al.,2011) in all the treatments. After 45 days of the bioassay, the pH values were significantly lower in the vermicompost with AWP compared to those with and without earthworms. This could be due to the release of volatile fatty acids because of higher organic matter (OM) degradation in these treatments. On the other hand, *E. fetida* mucus is added to the ingested materials, leading to the neutralization of the substrate (Pérez-Godínez et al., 2017). Moreover, earthworms have shown the ability to regulate the release of organic acids depending on the characteristics of the starting feedstock (Angst et al., 2019). The type of AWP also showed a significant effect on the pH, with plastics PET and PS showing the lowest pH values.

Table 22. Effect of the type of AWP material and *Eisenia fetida* presence in the physico-chemical and chemical parameters of the organic material obtained at the end of the bioassay (Paper 2, section 8.2.).

		pH	EC (dS m^{-1})	TOM (%)	TOC (%)	TN (%)	P (%)	K (%)	WSC (g kg^{-1})	C_{FA} (%)	C_{HA} (%)
***E. fetida* presence**	**Type of APW**										
Yes	No plastic t = 45d	7.42 a	5.42	52.9	23.7 b	2.24 a	0.81	1.28	8.83 a	3.66 c	3.58
	LDPE+LLDPE Black Film	7.24 a	4.81	53.6	26.2 c	2.31 ab	0.82	1.27	9.00 a	2.63 a	4.11
	LDPE+LLDPE Perforated Film	7.46 a	4.42	55.7	24.4 b	2.29 ab	0.82	1.16	8.36 a	2.47 a	3.27
	EPS Seedling	7.21 a	4.56	57.1	24.6 b	2.24 a	0.81	1.19	8.33 a	2.45 a	3.17
No	No plastic t = 45d	7.88 b	4.17	55.3	27.3 c	2.53 c	0.72	1.36	9.89 b	2.57 a	3.16
	LDPE+LLDPE Black Film	8.00 bc	3.26	52.4	24.3 b	2.38 b	0.83	1.08	10.3 b	3.31 b	4.96
	LDPE+LLDPE Perforated Film	8.20 c	3.20	54.4	24.0 b	2.34 b	0.81	1.12	9.23 b	3.01 b	3.76
	EPS Seedling	8.20 c	2.90	55.2	22.6 a	2.25 a	0.81	1.11	10.2 b	2.71 ab	3.87
Main effects											
E. fetida presence	Yes	7.35 a	4.92 b	54.4	24.8 b	2.26 a	0.81	1.22	8.41 a	3.01 b	3.52 a
	No	8.07 b	3.38 a	54.3	24.4 a	2.37 b	0.79	1.19	9.82 b	2.86 a	3.89 b
Type of APW	No plastic t = 45d	7.57 a	5.0 c	53.7 ab	24.9 b	2.33 b	0.78	1.28	8.83	3.32 c	3.42 a
	LDPE+LLDPE Black Film	7.61 a	4.04 b	53.0 a	25.3 b	2.34 b	0.82	1.18	9.65	2.97 b	4.53 b
	LDPE+LLDPE Perforated Film	7.83 c	3.81 a	55.0 ab	24.2 a	2.31 b	0.81	1.14	8.80	2.74 a	3.52 a
	EPS Seedling	7.70 b	3.73 a	56.1 b	23.6 a	2.24 a	0.81	1.16	9.26	2.58 a	3.58 a
Statistical significance											
	E. fetida presence	***	***	ns	***	**	ns	ns	***	***	*
	Type of APW	***	***	*	***	***	ns	ns	ns	***	**
	E. fetida x APW	***	ns	ns	**	***	ns	ns	***	***	ns

EC: Electrical conductivity, TOC: Total organic carbon, OM: Organic matter, Cw: Carbon water-soluble. ns, *, **, *** indicate not significant, statically significant at P≤ 0.05, P≤0.01, P≤0.001, respectively. Average values (n=3) in a column followed by the same letter are not significantly different at P< 0.05 (Tukeys and DMS test).

Table 23. Effect of the type of AWP material and *Eisenia fetida* presence in the physico-chemical and chemical parameters of the organic material obtained at the end of the bioassay (Paper 3, section 8.3.).

		pH	EC (dS m^{-1})	OM (%)	TOC (%)	TN (%)	P (%)	K (%)	WSC (g kg^{-1})	C_{FA} (%)	C_{HA} (%)
Main effects											
***E. fetida* presence**	Yes	7.47 a	4.66 b	53.8 a	26.5 a	2.36 a	0.74 a	1.16 a	8.19 a	2.52 a	3.23 a
	No	7.86 b	3.88 a	54.6 b	26.9 a	2.34 a	0.75 a	1.17 a	9.57 b	2.54 a	3.49 b
Type of AWP	LDPE	7.68 b	4.44 b	53.0 ab	28.4 b	2.35 a	0.72 b	1.19 b	7.66 ab	2.34 bc	3.06 b
	LLDPE	7.67 b	4.22 ab	53.8 ab	27.4 ab	2.33 a	0.76 bc	1.22 b	9.29 bc	2.75 b	3.96 b
	PET	7.49 a	4.23 ab	54.3 b	25.0 ab	2.40 a	0.61 a	0.78 a	7.61 ab	1.62 a	1.56 a
	PS	7.48 a	4.23 ab	54.0 ab	25.0 ab	2.35 a	0.63 a	0.79 a	7.18 a	1.58 a	1.88 a
	LDPE+LLDPE	7.73 bc	3.84 a	53.9 ab	24.6 a	2.31 a	0.81 c	1.21 b	9.09 bc	2.91 c	3.75 b
***E. fetida* presence**	**Type of APW**										
Yes	Control without AWP	7.48 ab	5.07 d	52.0 a	25.3 a	2.41 ab	0.76 a	1.24 a	8.22 a	2.93 a	3.75 a
	LDPE	7.50 ab	4.84 cd	52.1 a	27.8 a	2.44 ab	0.72 a	1.19 a	7.22 a	2.27 a	3.04 a
	LLDPE	7.52 b	4.42 bc	53.8 ab	27.3 a	2.30 a	0.74 a	1.22 a	8.83 a	2.64 a	3.70 a
	PET	7.27 a	4.66 c	53.2 ab	24.7 a	2.45 ab	0.64 a	0.81 a	7.36 a	1.64 a	1.28 a
	PS	7.42 ab	4.37 bc	52.9 ab	24.8 a	2.39 ab	0.60 a	0.73 a	6.82 a	1.71 a	1.64 a
	LDPE+LLDPE	7.37 ab	4.45 bc	55.0 b	25.2 a	2.29 a	0.72 a	1.24 a	8.35 a	2.65 a	3.47 a
No	Control without AWP	7.88 bc	4.17 bc	55.3 b	27.3 a	2.53 b	0.72 a	1.40 a	8.85 a	2.57 a	3.16 a
	LDPE	7.85 c	4.04 ab	54.0 ab	29.1 a	2.27 a	0.72 a	1.19 a	8.11 a	2.42 a	3.08 a
	LLDPE	7.82 bc	4.01 ab	53.9 ab	27.5 a	2.36 ab	0.78 a	1.28 a	9.73 a	2.86 a	4.21 a
	PET	7.71 bc	3.90 ab	55.4 b	25.3 a	2.31 a	0.59 a	0.75 a	7.86 a	1.61 a	1.85 a
	PS	7.53 b	4.08 b	55.1 b	25.3 a	2.31 a	0.65 a	0.85 a	7.54 a	1.43 a	2.11 a
	LDPE+LLDPE	8.10 c	3.22 a	52.8 ab	24.1 a	2.32 a	0.81 a	1.18 a	9.82 a	3.17 a	4.11 a
Statistical significance											
	E. fetida presence	***	***	*	ns	ns	ns	ns	*	ns	*
	Type of APW	***	***	***	***	ns	**	**	***	***	***
	E. fetida x APW	***	***	***	ns	**	ns	ns	ns	ns	ns

EC: Electrical conductivity, TOC: Total organic carbon, OM: Total organic matter, WSC: water-soluble carbon, C_{FA}: fulvic acid-like C; C_{HA}: humic acid-like C. n.s.: not significant P > 0.05; *, **, ***: significant at P ≤ 0.05, 0.01 and 0.001, respectively. Average values in a column followed by the same letter are not significantly different at P< 0.05 (Tukey-b post-hoc test).

The presence of *E. fetida* induced higher mean electrical conductivity (EC) values, with higher EC values also observed in the treatments with AWP and earthworms. The EC values obtained in all treatments with earthworms exceeded the threshold of 4 dS m^{-1}, which is a limiting value for plant cultivation in soilless crops (Lasardi et al.,2006), but remained within the recommended ranges for their use as organic amendments. On the other hand, EC values below 8 dS m^{-1} are considered adequate for earthworm growth and development (Rahimi and Karimi, 2016). The higher EC values observed in the treatment with *E. fetida* can be attributed to increased organic matter mineralization, leading to the release of ions (cations and anions), converting unavailable nutrients into more accessible forms, and the production of salts, ammonium, and inorganic (soluble salts) (Bernal et al., 2009). This behaviour coincides with a greater decrease in OM concentrations observed in the treatment with earthworms, which might also be explained by the ability of the earthworms to promote hydrolytic enzymes. These enzymes are not only linked to the C cycle (e.g., β-glucosidase or carboxylesterase) but are also related to other macronutrient cycles, such as the phosphorus cycle (phosphatase), which removes phosphate groups from OM (Nogales et al., 2008), or those related to N mineralization (urease, protease). The type of AWP also influenced this parameter, with the treatments containing a mixture of LDPE + LLDPE showing the lowest salinity mean values. The TOC content also experienced a significant reduction at the end of the bioassay compared to the initial feedstock, indicating accelerated mineralization of nutrients bound to OM. In the presence of AWP, a lower TOC reduction was observed, especially with LDPE + LLDPE, which could be indicative of a slight antimicrobial effect on the substrate. The type of AWP had a significant effect, with PET showing the highest OM mean contents at the end of the bioassay, possibly due to the different structure of this polymer, which could favour the retention of substances with an organic matrix (Fadare et al., 2019).

In general, the total nitrogen (TN) tended to increase in all treatments compared to the initial feedstock, both with and without earthworms, only finding a significant effect of the combined factors (*E. fetida* and AWP presence). Other studies on vermicomposting have reported an increase in TN due to the bioconversion process of waste decomposition by earthworms (Cynthia and Rajeskhumar, 2012). Additionally, microbial-mediated nitrogen transformation results in a further increase in nitrogen (Suthar and Singh, 2008).

Regarding P and K contents, no statistical differences were found when comparing treatments with and without earthworms. However, differences in P and K contents seem to be related to the type of AWP, with significantly lower content observed in PET and

PS. As observed in OM, the structural characteristics of these polymers could influence OM degradation. Previous studies have reported the capacity of polystyrene-based plastics to remain bound and retain substances with an organic matrix (Fadare et al., 2019), preventing their decomposition and the release of bonded nutrients. Thus, the obtained results suggest that the observed differences in NPK contents could be mainly associated with the type and composition of AWP.

The WSC values showed significant differences for both the presence of *Eisenia fetida* and the type of AWP plastic. The presence of earthworms significantly reduced the WSC contents, a result previously reported by other authors. This reduction is attributed to gut-associated processes and microorganisms consuming labile forms of organic matter as a carbon source for tissue formation during vermicomposting (Yadav and Garg, 2011). Regarding the effect of the type of AWP, treatments with LLDPE polymer in their composition generally presented higher values of WSC. The same behaviour was observed in treatments with and without earthworms for LLDPE. One potential reason for this is that the polymer backbone of LLDPE partially retains and bounds with the organic matrix, preventing its consumption. This finding agrees with a study by Chen et al. (2018) on the interaction of microplastics with the aromatic structure of dissolved organic matter.

The type of AWP had a significant effect on the humic and fulvic acid-like C contents, while the presence of *E. fetida* had a significant effect only on C_{HA}. In both parameters (C_{FA} and C_{HA}), the combination of *E. fetida* and AWP presence did not show a significant effect, while treatments with PET and PS again exhibited the lowest values. This reduction in humic compounds contrasts with what would be expected in the vermicomposting process. However, other studies about the presence of plastics in soil have reported a decrease in humic acid-like compounds due to the strong adsorption capability of polystyrene nanoplastic particles (Velzeboer et al., 2014; Cai et al., 2018).

The Dehydrogenase/ water soluble carbon ratio (DHE/WSC) links microbial activity with the amount of easily metabolized organic matter. In our study, the initial feedstock showed a high amount of WSC (18.9 g/kg), which could lead to a quick increase in degradative and hydrolytic activity by microorganisms in the feedstock and the gut microbiome of earthworms. In all treatments (LLDPE + LDPE and EPS), the vermicompost WSC decreased at the end of the bioassay, suggesting substrate depletion and indicating the correct evolution of microbial activity and the biotransformation of available organic matter into more stable molecules. The DHE/WSC

ratio showed remarkable differences depending on the factors used in the statistical analysis, namely, the type of plastic and the presence of earthworms. The LLDPE + LDPE black film and LLDPE + LDPE perforated film without earthworms had the highest values for that parameter (8.06 and 8.00, respectively).

The particle size exerted a significant effect on the physicochemical characteristics of vermicompost like the effect of UVC pre-treated plastics. Changes in particle size (MP) may have indicated alterations in the polymeric structure of AWP materials, rendering them more readily available for biological attack (Shah et al., 2008; Urbanek et al., 2021).

Table 24. Significant effect produced by the presence of AWP on the physicochemical characteristics in the vermicompost obtained in the presence of *E. fetida*.

Significant effect		Featured AWPs
pH	↓	PET & PS
EC (dS m^{-1})	↓	LDPE + LLDPE, LDPE + LLDPE (Black Film and Perforated Film), PS, LLDPE & EPS
MOT (%)	↑	LDPE + LLDPE, PS, LLDPE & EPS
TOC (%)	↓	LDPE + LLDPE, LDPE + LLDPE (Perforated Film) & EPS
TN (%)	=	LLDPE, LDPE + LLDPE & EPS
P (%)	↓	PET & PS
K (%)	↓	PET & PS
WSC (%)	↑	LLDPE & LDPE + LLDPE
C_{FA} (%)	↓	LDPE + LLDPE (Black Film and Perforated Film) & EPS
C_{HA} (%)	↓	PET & PS
DHE/WSC	↓	LLDPE+LDPE & EPS

*↓: Decrease in value, ↑: Increase in value and =: No change in value.

Regarding the heavy metal content in the vermicompost, although concentrations of Cu, Zn, Cd, and Co slightly increased after the vermicomposting process, likely due to organic matter degradation and subsequent volume reduction, the final levels met the European Union Eco-label requirements for ecological production (Regulation (EU) 2019/1009). Therefore, they can be used as organic amendments in agriculture. The concentrations of Cr and Ni were below detection limits (<0.01 mg kg^{-1}).

4.4.2. Biochemical indicators

The results of the vermicompost analysis revealed a significant difference in **carboxylesterase** (CbE) activity when compared to the control treatment without plastic. This induction in CbE production was observed in all treatment (except LLDPE + LDPE), with and without earthworms. Notably, in all cases under study, the treatments without *E. fetida* presence (compost treatment) exhibited greater sensitivity to the observed increase in CbE activity, LDPE, LLDPE, LDPE + LLDPE (Black and Perforated film), PS, PET and EPS, during the assay led to a significant increase in CbE activity compared to the control treatment without plastic, with a mean increase of 39.7%. When comparing the control treatment without AWP with the presence and absence of earthworms, an increase in CbE activity is evident (Figure 25).

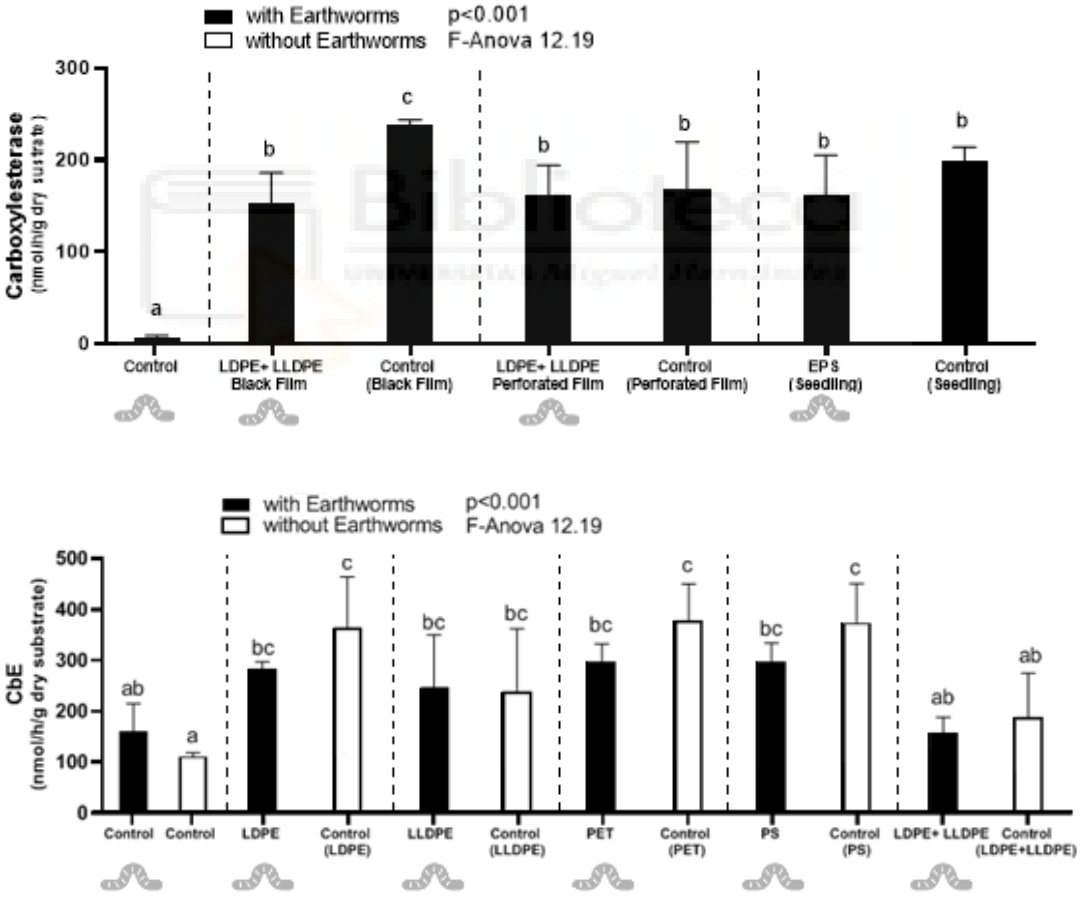


Figure 25. Carboxylesterase (CbE) activity as biochemical indicators of the effects of the presence of AWP in Vermicompost obtained.

This increase coluld be attributed to the fact that this esterase is primarily secreted in the intestinal lumen of the earthworms themselves (Sánchez-Hernandez et al., 2009). Consequently, earthworm castings may act as a source of stable and active CbE (Sánchez-Hernández et al., 2015). The observed increase in CbE activity in the presence of plastic materials may be linked to cellular damages, potentially resulting in changes in energy consumption to counteract the effects imposed by oxidative stress or other mechanisms (Rodríguez-Seijo et al., 2018).

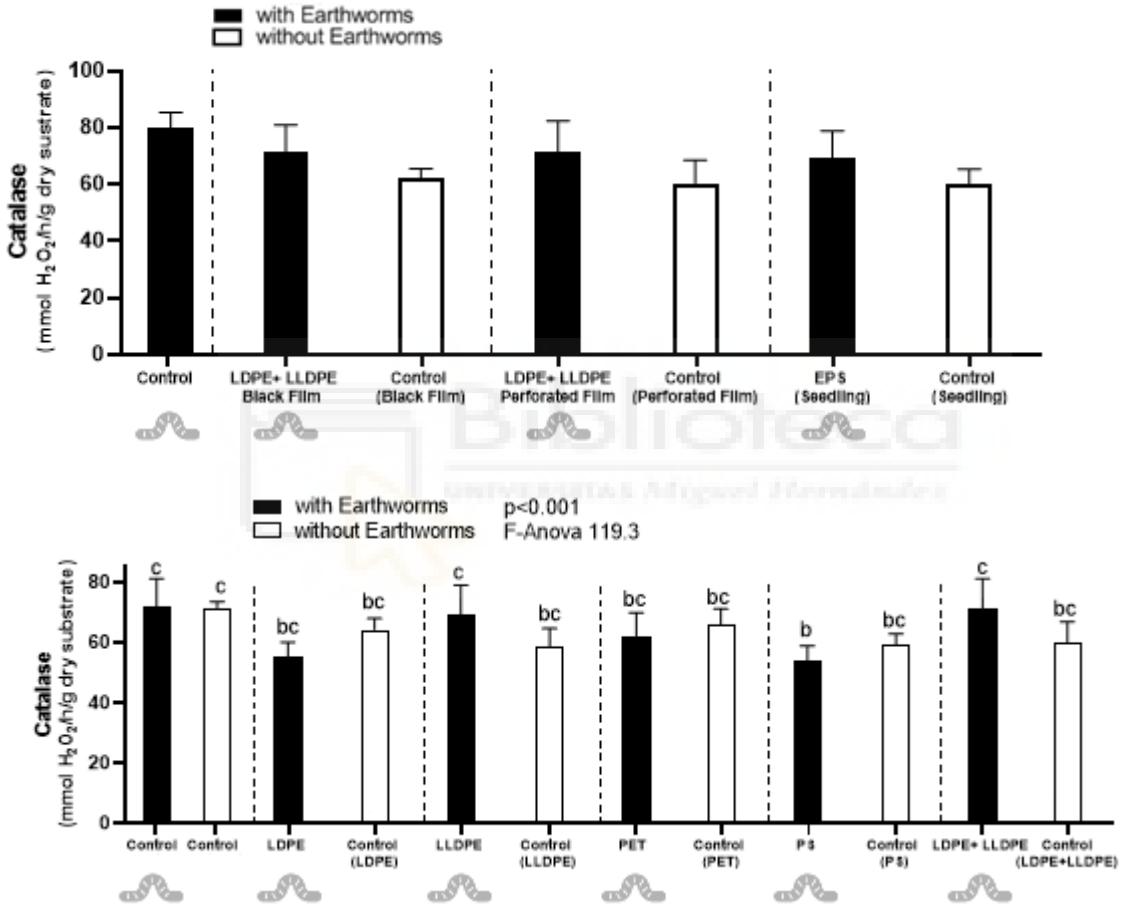


Figure 26. Catalase activity as biochemical indicators of the effects of the presence of AWP in vermicompost obtained.

The **catalase** enzyme plays key role in the antioxidant system, responding to oxidative stress and preventing oxidative damage (Giulia et al., 2012). Generally, a slight inhibition of catalase (CAT) activity was observed in all treatments with AWP presence compared to the control treatment, in both cases, with and without earthworm. In the presence of earthworms, only the treatments with LDPE, PET and PS showed statistically lower values than the control treatment, while the rest of the AWP treatments exhibited values

statistically similar (Figure 26). A potential reason for the observed decline at these low levels could be the ability of certain plastic additives to act as CAT inhibitors. Additives such as hydroxylamine and metallocenes are commonly used as UV and light stabilizers in the film manufacturing process, while resorcinol serves as an efficient gas barrier for several polymers (Rodríguez-Seijo et al., 2018). These compounds have an affinity for binding to the catalase enzyme site, leading to its permanent inactivation (Pritchard, 1998; Rodríguez-Seijo et al., 2018). Thus, the presence of AWP in the tested proportion (1.25% f. w.) appears to result in a partial inhibition of CAT production, but it does not induce a clear detoxifying response in *E. fetida*.

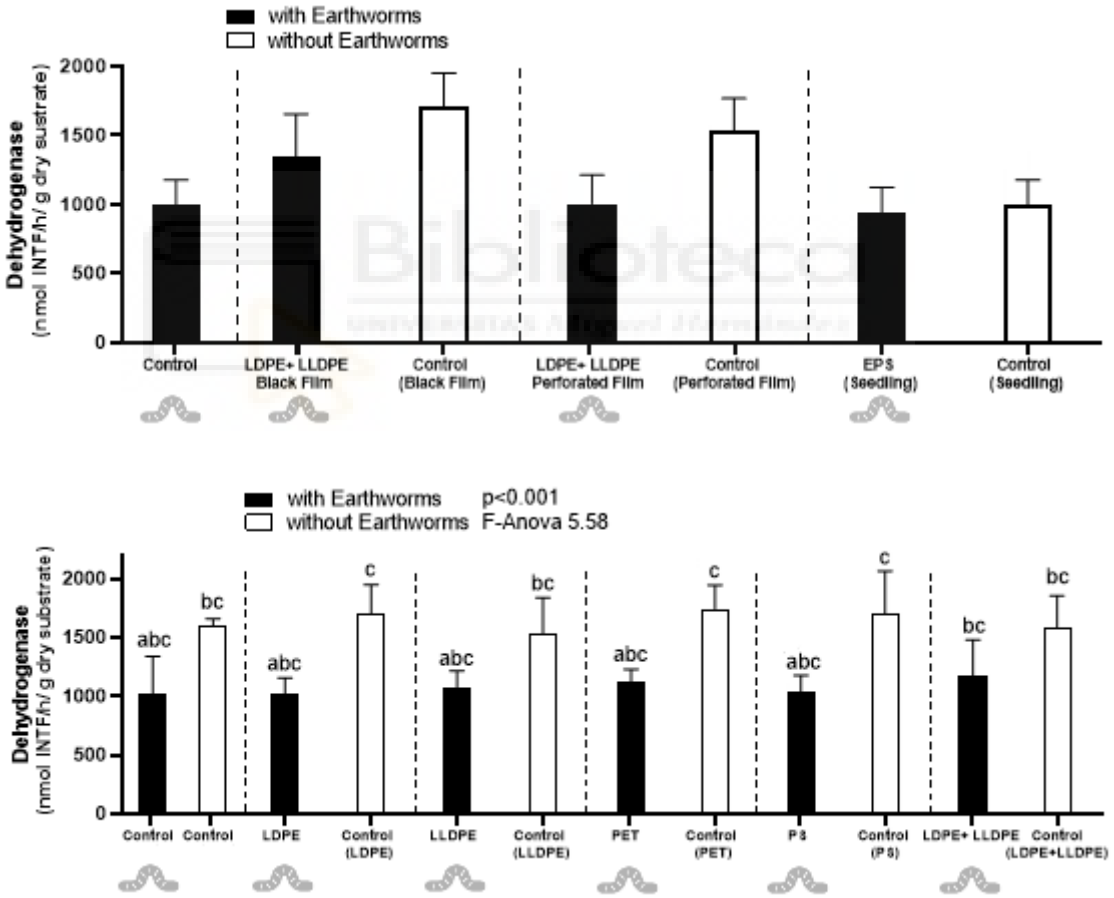


Figure 27. DHE activity as biochemical indicators of the effects of the presence of AWP in Vermicompost obtained.

DHE activity is directly linked to the biological oxidation of organic matter. The type of AWP seemed not induce a significant effect on DHE activity in the presence of *E. fetida*, with similar values observed as in the control treatment. However, it is worth noting a

slight increase in DHE activity observed in the compost treatment compared to the vermicompost, especially with LDPE + LLDPE black film, LDPE + LLDPE perforated film and with EPS with 24.9, 37.3, 53.3 % of increase respectively. These results suggest that the digestive system of earthworms was capable of breaking down the organic matter in the feedstock with AWP, leading to an increase in the particle surface-to-volume ratio and consequently maintaining the number and activity of microorganisms. Moreover, the higher DHE activity observed may be attributed to a lower level of stabilization achieved in these samples (Figure 27). This is supported by the higher WSC content remaining in samples without earthworm presence (Annex 7.2 y 7.3).

On the other hand, slight differences were found in the enzyme activities considered as a function of AWP particle size (film debris or MP). The only enzyme activities affected were CbE and DHE, with no significant effect on CAT activity. Furthermore, film debris seems to induce a lower inhibition of CbE and DHE activities than MP. The lower enzymatic activities observed in MPs could be related to a lower biological oxidation activity of the total organic carbon (TOC) of the substrate due to the potential release of toxic compounds derived from the plastic materials when they are physically and/or chemically modified. Thus, significant differences found in enzymatic activities related to changes in size and/or composition may be evidence for the presence of plastic-derived compounds that may induce adverse effects.

Table 25. Significant effect produced by the presence of AWP on the Enzymatic activity in the Vermicompost obtained in the presence of *E. fetida*.

Significant effect		Featured AWPs
CbE (nmol/h/g dry subsatrate)	V ↑	LDPE, LLDPE, LDPE + LLDPE (Black and Perforated film), PS, PET & EPS
CAT (mmol H_2O_2/h/g dry substrate)	V ↓	LDPE, PET & PS
DHE (nmol INTF/h/g dry substrate)	V ↑	LDPE + LLDPE

*↓: Decrease in value, ↑: Increase in value and =: No change in value, B = Biofilm and V = Vermicompost.

4.5. Use of advanced strategies to mitigate the presence of plastics in the vermicomposting media.

The microorganisms have the ability to degrade or break down complex organic matrix by various biochemical and enzymatic mechanisms, allowing the degradation of complex compounds such as lignin, polyphenols or plastic, thus contributing to the natural cycle of the decomposition of organic fluxes materials. In the RECOVER project, where this PhD is included, a biotechnological cascade has been developed to mitigate the effect caused by the presence of plastics in the vermicomposting media using beneficial microorganisms (Figure 28). On the other hand, insect larvae (Ep*hestia kuehniella, Galleria mellonella, Plodia interpunctella* and *Tenebrio molitor*), anecic earthworms (*Lumbricus terrestris*), beneficial microorganisms have been developed in each environment contaminated by AWP and new AWP-biodegrading enzymes have been designed.

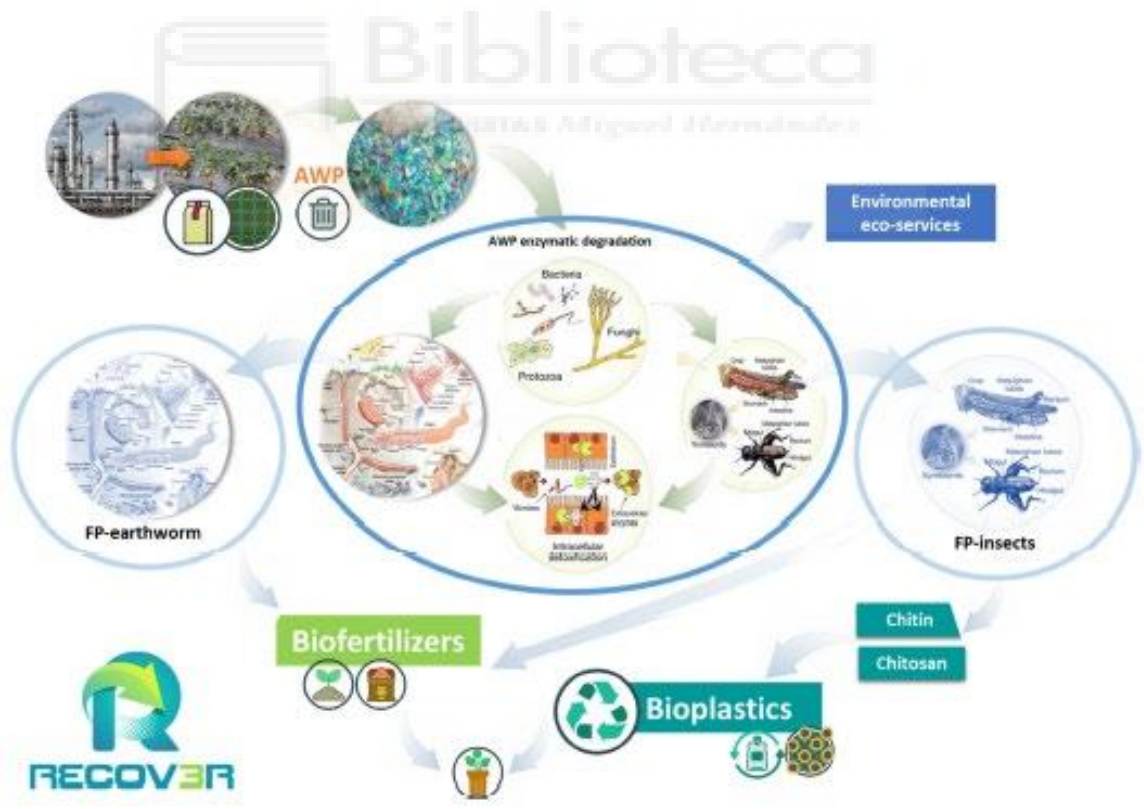


Figure 28. Graphical abstract of Recover project and the key-issue to produce pre-treated AWP to biotic degradation

The RECOVER matrix used was an organic waste fluxes polluted by plastics. These media are normally associated to mixed AWP collection or mechanical-biological treatments of municipal wastes, where impurities of plastics are increasing into them. This waste flux is rich in organic matter (TOM>30% f.w.), high humidity (water content 70% f.w.) and we established the plastic concentration at 1.25% f.w. Microbial consortia were identified, isolated, and cultured from contaminated environments by Almeria University research team. Microbial consortia were studied and defined for *Eisenia fetida* by UAL including two main groups of microorganisms EF-EXO-PMC with polymer-degrading capabilities and EF-ENDO-PMC obtained from the gut-microbiome of *E. fetida* exposed to AWP with probiotic capacities.

The composition of ENDO-PMC was:

- *Bacillus licheniformis S-ALME2-B2*,
- *Pseudomonas putida ALME2-B2* and
- *Bacillus sonorensis ALME2B3.*

The composition of EXO-PMC was:

- *Bacillus subtilis RBM2* and
- *Pseudomonas putida REBP7*.

4.6. Effects of PMC inoculation on *Eisenia fetida*

To evaluate the biotechnological cascade including PMC solutions, mesocosm bioassays were performed in our standardized vermicomposting system. The bioassay was developed using a mesocosm-scale, inoculated with EXO, ENDO, and MIX-PMC (vg. EXO+ENDO PMC) to assess the performance of beneficial microorganisms in producing fortified earthworms, improve their capabilities, and enhance the process in the presence of AWP (Paper 4, section 8.4). In this context, the most relevant results are shown below.

4.6.1. Survival

The evaluation of probiotics consortium inoculated feedstock (ENDO-PMC) revealed significantly improve in *E. fetida* survival if compared with control. In EXO-PMC inoculated feedstock, it was found that they had no detrimental effect on survival compared with control treatment without microorganism inoculation. Finally, the MIX-PMC (ENDO+EXO) showed the higher survival rate (in presence of LDPE and specially for Mix plastic) (Figure 29). These findings differ from those reported in a study of vermicomposting of sewage sludge with presence of microplastic of PP and HDPE where no significant effect on survival were found (Ragoobur et al., 2022). In concordance with this, Judy et al. (2019) in incubation of *Eisenia fetida* reported no significant differences in earthworm survival, growth or reproduction between the municipal organic waste amended soil controls and the municipal organic waste amended soil with microplastics (HDPE and PET) treatments. This corroborates the hypothetical improvement of *Eisenia fetida*, and it can be assumed that a synergetic effect was produce in both: a) Gut-associated microorganism of earthworms (ENDO-PMC) with the subsequent improve in their physiological activity and b) in inherent feedstock microorganism activity by EXO-PMC.

4.6.2. Morphological effect

Regarding to loss of body weight, in general the inoculation with the different microbial consortia tested were able to maintain lower loss of weight of *E fetida* than control treatment, but without significant differences. The treatment with mean lower weight reduction corresponds with EXO-PMC. After 30 days of exposure, two treatments showed a weight reduction higher to 20% (LDPE-No PMC and Mix Plastic-Mix C), which could be considered a harmful effect on metabolic process. We found statistical differences for the inoculation with EXO-PMC, where a lower weight reduction was observed by LDPE and Mix Plastic exposure treatments (-9.5 and -1.4 %, respectively) than No plastic treatment (-12.6 %). No PMC and Mix C also exhibited statistical differences, but no clear relationship could be established between this observed effect and the action of PMC (Figure 29).

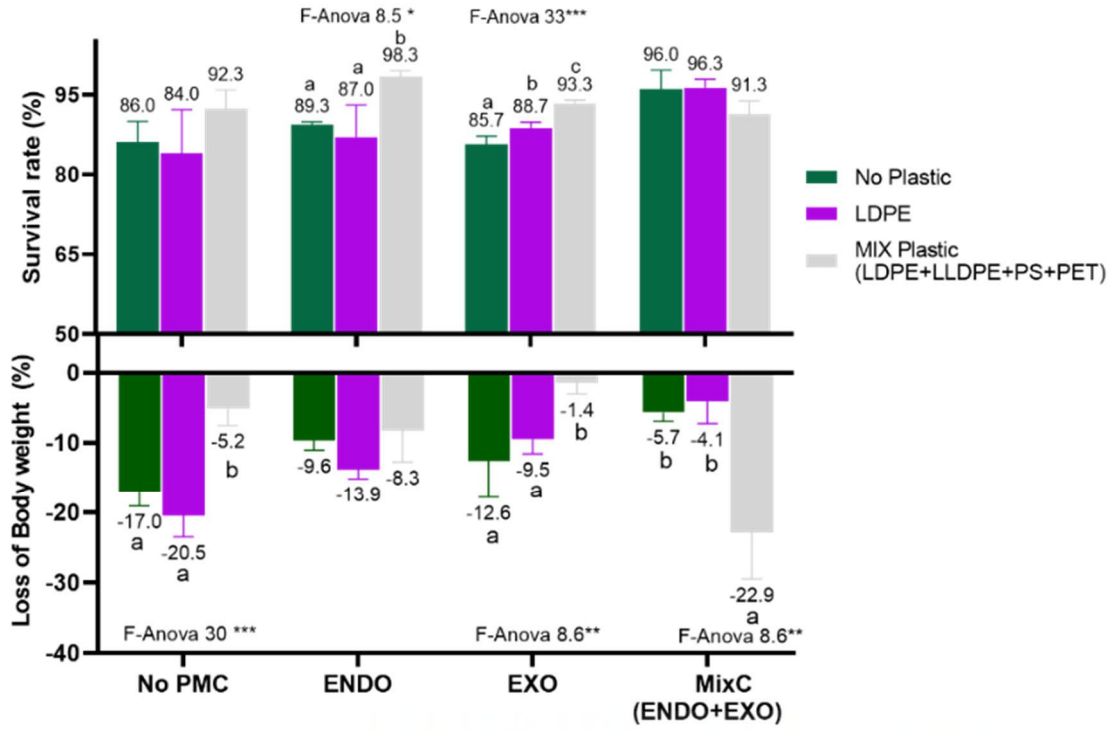


Figure 29. *E. fetida* survival (%) and body weight variation (%) depending of the type of consortium and added plastic materials

4.6.3. Response of biomarkers

To identify the oxidative stress that the earthworm *Eisenia fetida* may suffer, a series of biomarkers are used: CbE, GSH, GSGG, Lipid Peroxidation and AChE (Figure 30 and Table 26). In addition, the IBRv2 index have been used to assess the integrate biomarkers responses (described in material and methods of Annex 7.4.).

The results suggest that for these two types of AWP (LDPE and MIX) in the absence of PMC there was no induction of molecular mechanisms in *E. fetida* that caused an increase in CbE activity (Figure 30). These results are consistent with the behavior of some AWPs, which showed CbE activity values close to the control (such as LDPE, LLPDE, LLDPE + LDPE and EPS, analysed in section 5.3.3). After application of the PMCs there was also no induction of their effect on *E. fetida* (Ai values, Figure 30), but there were differences between the types of PMCs used, with a significant increase in inoculation with ENDO-PMC (Table 26).

As for lipid peroxidation activity, in the absence of PMC, we found some induction of activity in the presence of MIX-AWP. This may be explained by the fact that by having a

varied chemical composition, it may be able to harm *E. fetida* through different regions of exposure. This is consistent with other studies (Wang et al., 2020; Chen et al., 2020; Jiang et al., 2020). When inoculated with EXO and MIX-PMC, lipid peroxidation activity is significantly increased compared to non-inoculation. We hypothesize that the reasons for this could be that the presence of microorganisms external to *E. fetida* may be altering their immunity (Table 26 and 27).

AChE activity is a biomarker of neurotoxicity, breaking down acetylcholine to mitigate the neurotoxic effects of pollutants in various species (Zhang et al., 2020). LDPE and MIX-AWP stimulated the neurotoxicity response in *E. fetida*, as reflected in the induction of AChE activity. The inoculation with PMC in the presence of AWP exerted a specific positive regulatory effect on neurotoxins, decreasing AChE activity close to control activity (highlighting EXO-PMC for both AWP).

As shown in Figure 30, the index clearly showed that exposure to MIX-AWP plastic induced oxidative stress in earthworms, as evidenced by high Ai scores for both GSGG and GSH activities. The result obtained in the Ai score and its star graph representation revealed a differential behavior depending on the PMC inoculated (Figure 30). On the one hand, inoculation with ENDO-PMC produced an inhibitory effect on *E. feida* tissue in both the LDPE and MIX-AWP treatments, reflected in GSGG and GSH activity. On the other hand, inoculation with EXO-PMC for the LDPE exposure showed a similar behavior to the control treatment without inoculation and for the MIX-AWP exposure, oxidative stress levels were still induced.

It can be concluded on the one hand that the presence of LDPE without PMC inoculation exerted a negative effect especially at the neurotoxic activity in *E. fetida*. The inoculation with PMC in general inhibited the negative effect caused by the presence of AWP, highlighting the MIX-PMC integrating IBRv2 values. On the other hand, the presence of MIX-AWP without PMC inoculation seems to indicate that it exerted a negative effect on oxidative stress, tissue or immune damage and neurotoxicity in *E. fetida*. The inoculation with PMC in general inhibited these effects on earthworms, with ENDO-PMC integrating IBRv2 values standing out.

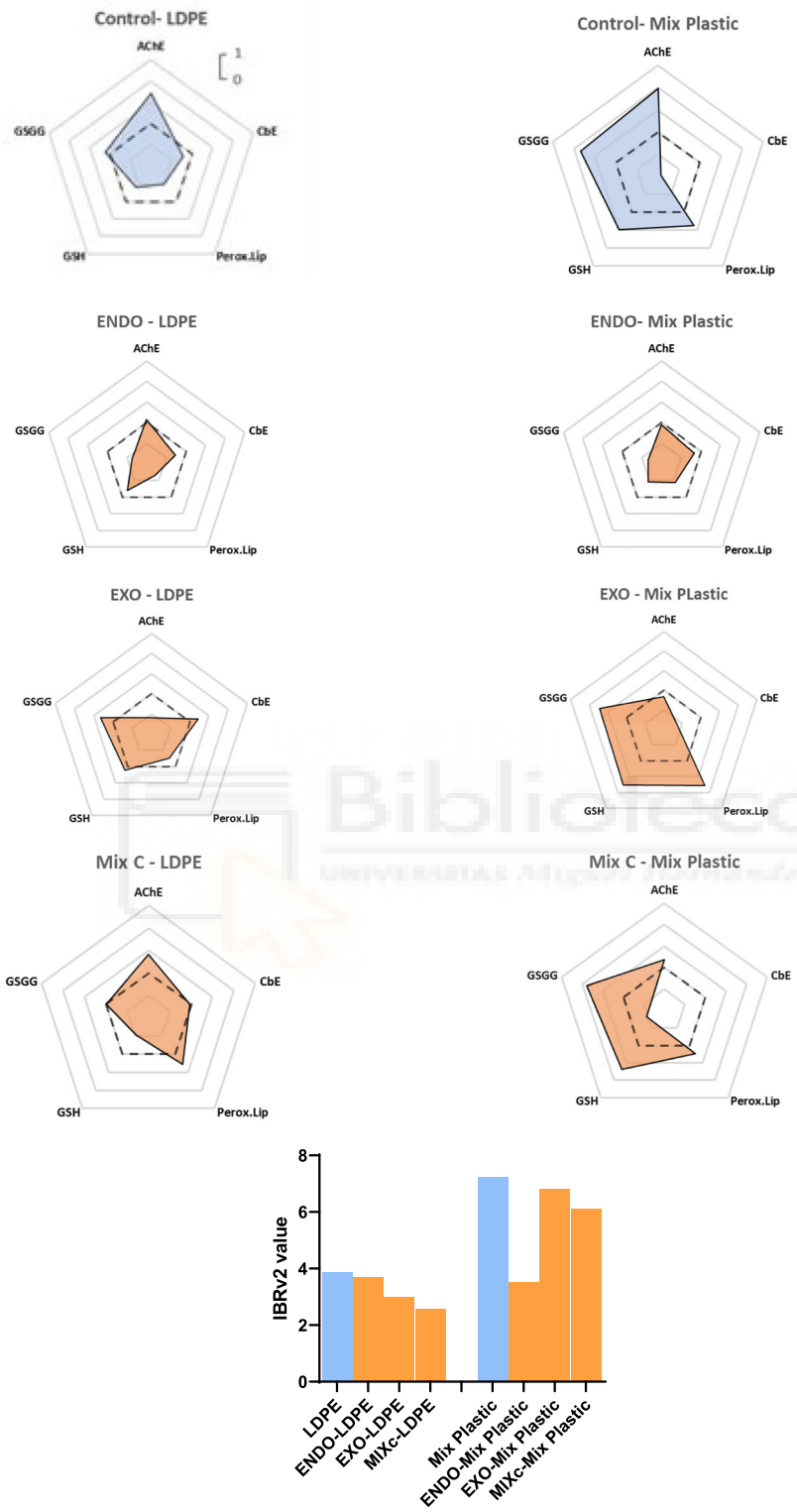


Figure 30. Star plots of the A_i score (deviation index) of biomarkers measured in muscle tissue of *Eisenia fetida*. Dotted lines in star plots indicate the reference values correspond to control earthworms (without addition of PMC or plastic). Bar plot shows the integrated biomarker response index (IBRv2) values calculated for each plastic type.

*AChE: Acetylcholinesterase; GSH: Reduced glutathione; GSGG: Oxidized glutathione; Perox. Lip.: Lipid peroxidation; CbE: Carboxylesterase activity.

Table 26. Statistical effect of type of PMC on *Eisenia foetida* biomarkers

	CbE (µmol h^{-1} mg^{-1} protein)	GSH (nmol mg^{-1} protein)	GSGG (nmol mg^{-1} protein)	PerLip (µgMDA mg^{-1} protein)	AChE (nmol min^{-1} mg^{-1} protein)
Type of PMC					
No PMC	487 ab	0.53 a	0.81 b	4434 a	361 c
ENDO	516 b	0.71 b	0.71 a	4192 a	264 b
EXO	478 ab	0.86 c	1.14 c	5250 b	213 a
MIXc	444 a	0.93 c	1.36 d	5831 c	276 b
F-anova	3.3*	44.8***	109***	21.2***	42.2***
Statistical significance					
Type of PMC	*	***	***	***	***
AWP X PMC	6.5***	22.1***	57.4***	16.1***	ns

*, **, *** indicate not significant, statically significant at P≤ 0.05, P≤0.01, P≤0.001, respectively. Average values (n=3) in a column followed by the same letter are not significantly different at P< 0.05 (Tukeys-B and Duncan post-hoc test)

Table 27. Significant effect produced by the presence of PMC with AWP on *E. Fetida*: survival, body weight and response of biomarkers.

Significant effect			Featured PMCs
Morphological response	Survival (%)	↑	LDPE → EXO-PMC MIX-AWP → ENDO & EXO-PMC
	Body weight (%)	↑	LDPE → EXO-PMC MIX-AWP → EXO-PMC
Biomarkers response	CbE (µmol h^{-1} mg^{-1} protein)	↓	LDPE → ENDO & EXO-PMC MIX-AWP → ENDO, EXO & MIX-PMC
	Lipid peroxidation (µgMDA mg^{-1} protein)	↓	LDPE → ENDO & EXO-PMC MIX-AWP → ENDO & MIX-PMC
	AChE (nmol min^{-1} mg^{-1} protein)	↓	LDPE → ENDO & EXO-PMC MIX-AWP → ENDO, EXO & MIX-PMC
	GSGG (nmol mg^{-1} protein)	↓	LDPE → ENDO & MIX-PMC MIX-AWP → ENDO-PMC
	GSH (nmol mg^{-1} protein)	↓	LDPE → ENDO & MIX-PMC MIX-AWP → ENDO-PMC

*↓: Decrease in value, ↑: Increase in value and =: No change in value.

4.7. Effects of the inoculation with PMC in vermicompost obtained.

In this section we will show the results of the effect of PMC inoculated in the presence of AWP on the vermicompost obtained. The results have been published in the Paper 4 (Annex 7.4.).

4.7.1. Agronomic quality

The effect of PMC inoculation in mesocosm trials is described below. Regarding pH, a slight decrease was observed at the end of the trial in all treatments, but with no significant differences among them. The pH values at the end of the bioassay remained within a suitable range for earthworm activity and microorganism growth (5.5-8.5) (Yadav et al., 2011) in all treatments. If we examine the EC results, the values obtained significantly increased at the end of the trial in treatments with AWP presence and the control treatment. The increase in EC during the vermicomposting process has been reported by other authors (Khali and Sanaa, 2009; Fernández-Gómez et al., 2010). The reason for the EC increases in the earthworm treatment could be attributed to the greater mineralization of organic matter, releasing nutrient ions and soluble salts (Huang et al., 2017). Regarding parameters that could provide information about the performance of decomposition process of organic matter mediated by *E. fetida*, the MOT, TOC, and C/N, are affected to different aspects. The presence of AWP affects the TOC and MOT at the end of the trial, finding values higher than the control. When applying PMC, no significant differences were found for the C/N ratio, but there were differences for MOT and TOC. The application of EXO and MIX-PMC favours a significant decrease in MOT content. This behaviour is also observed in TOC content. As for nutrient content (P and K), there are slight significant differences at the end of the PMC application trial. The application of ENDO and EXO-PMC favours obtaining a product with slightly significantly higher P and K values than the control. There are no significant differences due to the presence of plastic, indicating that it does not seem to affect the nutritional content of the obtained vermicompost.

Table 28. Results of the physico chemical effects of microplastic presence depending of PMC inoculated or type of plastic materials

	pH	EC (dS m^{-1})	MOT (%)	TOC (%)
Type of AWP				
No AWP	8.06	4.28	37.2 a	22.9 a
LDPE	8.01	4.39	38.8 c	24.2 b
Mix-plastic	8.04	4.39	38.3 b	25.0 c
F-anova	*ns*	*ns*	*66.2***	*25.7***
Type of PMC				
No PMC	8.02	4.25	38.9 c	23.2 a
ENDO	8.02	4.37	39.3 d	25.0 c
EXO	8.05	4.39	37.7 b	24.3 bc
MIXc	8.05	4.41	36.4 a	23.6 ab
F-anova	*ns*	*ns*	*128***	*11.5***
Statistical significance				
Type of AWP	*ns*	*ns*	***	***
Type of PMC	*ns*	*ns*	***	***
AWP X PMC	*	***	***	***
	TN (%)	**C/N**	**P (%)**	**K (%)**
Type of AWP				
No AWP	1.97	11.6 a	0.53 c	0.88 b
LDPE	1.95	12.4 b	0.47 a	0.83 a
Mix-plastic	1.99	12.6 b	0.50 b	0.86 ab
F-anova	*ns*	*10.3****	*12.0****	*4.41**
Type of PMC				
No PMC	1.95	11.9	0.47 a	0.80 a
ENDO	2.01	12.5	0.53 b	0.92 b
EXO	1.95	12.4	0.52 b	0.90 b
MIXc	1.97	12.0	0.49 a	0.82 a
F-anova	*ns*	*ns*	*9.5****	*17.0****
Statistical significance				
Type of AWP	*ns*	***	***	*
Type of PMC	*ns*	*ns*	***	***
AWP X PMC	***	*ns*	*	*ns*

Table 29. Significant effect produced by the presence of PMC with AWP on the physicochemical characteristics in the vermicompost obtained in the presence of *E. fetida*.

Significant effect		Featured AWPs
pH	=	ENDO, EXO and MIX-PMC
EC (dS m^{-1})	=	ENDO, EXO and MIX-PMC
MOT (%)	↓	MIX-PMC
TOC (%)	↓	MIX-PMC
C/N	=	ENDO, EXO and MIX-PMC
TN (%)	=	ENDO, EXO and MIX-PMC
P (%)	↑	ENDO and EXO-PMC
K (%)	↑	ENDO and EXO-PMC

*↓: Decrease in value, ↑: Increase in value and =: No change in value.

4.7.2. Biochemical indicators related to vermicompost

In the results obtained for the different enzymatic activities of the vermicompost by PMC presence, we found significant differences in the activity of the dehydrogenase enzyme (DHE) according to the type of plastic material exposure after t = 30 days. The presence of the MIX plastic led to a slight decrease in DHE activity in the vermicompost with respect to Control, this being the only treatment showing significant differences. This decrease in DHE enzyme activity with MIX plastic could be due to a worse effect in rate of bioxidation of organic matter with consequent reduction in DHE activity.

Table 30. Effect of the main variables (type of AWP and PMC addition) on biochemical indicators related to vermicompost

	CbE (nmol/h/g dry subsatrate)	CAT (mmol H_2O_2/h/g dry substrate)	DHE (nmol INTF/h/g dry substrate)
Type of AWP			
No AWP	164	34.2 a	1035 b
LDPE	166	37.0 b	1037 b
MIX plastic	163	34.4 a	944 a
F-anova	ns	5.9**	11.3***
Type of PMC			
No PMC	152 a	38.5 c	985
ENDO	167 b	35.4 b	1017
EXO	170 b	30.8 a	1002
MIXc	168 b	36.0 bc	1016
F-anova	4.0*	18.0***	ns
Statistical significance			
Type of AWP	ns	**	***
Type of PMC	*	***	ns
AWP X PMC	6.4***	9.5***	6.9***

CbE= carboxylesterase,*, **, ***, indicate not significant, statically significant at P≤ 0.05, P≤0.01, P≤0.001, respectively. Average values (n=3) in a column followed by the same letter are not significantly different at P< 0.05 (Tukey's-B and Duncan post-hoc test)

As regard to Catalase, also found significant differences from result of enzyme catalase activity according to the factor type of microbial consortium. The presence of the EXO inoculum led to a significant decrease in CAT activity in the vermicompost with respect to No PMC treatment. This decrease could be associated with a lower effective interaction between the inoculated EXO microorganism and the degradative activity in the earthworm gut. This result agrees with previous studies (Blesa Marco et al., 2023) where found an inhibitory effect in CAT activity during vermicomposting process exposure to PET and LDPE + LLDPE microplastic. In addition, Samal et al., 2023

reported a significantly decrease of hepatic catalase activity of human in presence of Polyethylene bags microplastics.

In opposite trend, for carboxylesterase (CbE) activity, in general, the three different inoculum consortia seemed to induce an increase in the values obtained. The exposure to LDPE and MIX plastic not induced higher change in CAT and CbE activity if compare with Control treatment.

Table 31. Significant effect produced by the presence of PMC with AWP on the Enzymatic activity in the Vermicompost and Biofilm obtained in the presence of *E. fetida*.

Significant effect		Featured AWPs
CbE (nmol h^{-1} g^{-1} dry substrate)	↑	ENDO, EXO and MIX-PMC
CAT (mmol H_2O_2 h^{-1} g^{-1} dry substrate)	↓	ENDO, EXO and MIX-PMC
DHE (nmol INTF h^{-1} g^{-1} dry substrate)	=	ENDO, EXO and MIX-PMC

*↓: Decrease in value, ↑: Increase in value and =: No change in value.

4.8. Persistence of PMC inoculum in *Eisenia fetida* and vermicompost obtained in AWP polluted systems

At the end of mesocosm-scale trials, surviving earthworms exposed to LDPE and Mix plastic in presence or absence of probiotics consortium (EXO and ENDO-PMC) were collected and their gut microbiome subjected to metataxonomic analysis. The result obtained in quantification of Total Bacteria (data shown in Anex 7.4) showed as the load in colony forming units in control treatment without inoculation of PMC no significant difference was found when earthworms were exposed to plastic (LDPE and Mix plastic), whilst in ENDO, EXO and Mix c (ENDO+EXO) the exposed to both plastic lead to a great Gut-microbiome load at the end of bioassay with statistical differences. Except for ENDO+EXO inoculated earthworms exposed to Mix plastic, the load of *Pseudomonas spp* (Fig 31) in gut-microbiome of earthworms exposed to LDPE and MIX-plastic also became significant higher if compared with Earthworms without inoculum application. Finally, the presence of LDPE induced the great extent the increase in abundance of the *Bacillus spp*. In the case of exposure to plastic mixture, a significant increase was also observed when compared to the control, although this increase effect was less evident.

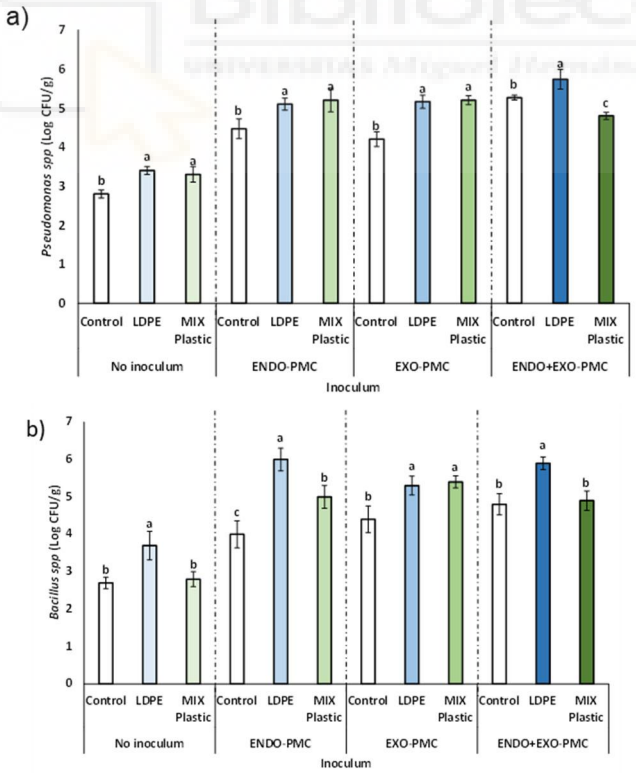


Figure 31. Logarithm of colony forming units (Log CFU g - 1) of the digestive tract of *E.fetida* obtained after PMC (ENDO,EXO and Mix C ENDO+EXO-PMC) inoculation in plastic spiked (LDPE and MIX Plastic) feedstock. No inoculum and no plastic controls were set as negative controls for PMC inoculation and plastic presence,respectively. The results represent the means (n = 3) ± SD (vertical bars). The bars illustrate (a) *Pseudomonas spp* and (b) *Bacillus spp*.

In order to trace the presence and load of inoculum in the vermicompost obtained, at the end of bioassay metataxonomic characterisation was also done in vermicompost for each experimental conditions tested (Fig 32). The inoculation with the probiotics had a clear impact on increase of *Pseudomonas* and *Bacillus spp*, especially when vermicompost were exposed to plastic materials. The results validated the successful colonization of the vermicompost by two type of consortium bacteria after 30 days, which increased in all sample with respect to initial load inoculated. Also, the fact that the levels for the two microorganisms where much higher in the inoculated than in the non-inoculated vermicompost, of the order of 2 log units and 1.5 log units for *Pseudomonas* and *Bacillus spp*, respectively. This increment in count of colony forming units in inoculated sample with respect to no inoculated could indicate that this load increase come from the microorganisms inoculated and not from indigenous *Pseudomonas* or *Bacillus* in Initial vermicompost.

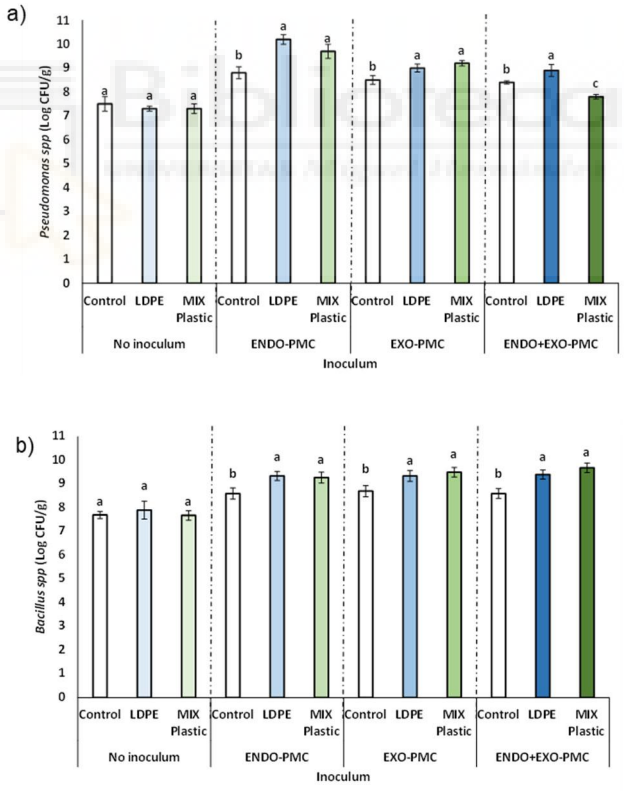


Figure 32. Logarithm of colony forming units (Log CFU g - 1) of the plastic spiked biowaste (LDPE and MIX Plastic) obtained after PMC (ENDO,EXO and Mix C ENDO+EXO-PMC) inoculation. No inoculum and no plastic controls were set as negative controls for PMC inoculation and plastic presence,respectively. The results represent means (n = 3) ± SD (vertical bars). Bars illustrate the (a) *Pseudomonas spp* and (b) *Bacillus spp* in each experimental condition tested. Homogeneity groups are represented respecting to each inoculum.

4.9. Biodegradation performance of LDPE and PS using PMC inoculation into the vermicomposting systems

The *m/z* ions determined by TGA-MS could be grouped for each type of plastic, from PE the *m/z* 41,43 and 56 ions produce the highest intensity values. These ions corresponding to C_3H_5, C_3H_7, and C_4H_8, respectively and have been related with thermal degradation products of PE (Dümichen et al., 2015). On the other hand, the highest signals for PS corresponding with *m/z* 78 and 104, related to benzene (C_6H_6) and styrene (C_8H_8), respectively (Qureshi et al.,2020). In our study, the Initial (t 0d) and final control samples (un-spiked organic samples) were also analysed and compared to eliminate interferences from the natural evolution of the organic sample matrix. Regarding to control treatment without plastic added an increase in MS signal intensity was noticed for *m/z* 78 (13 %), while minimal change was found for *m/z* 41,43,56,104 with ±2 % of signal change at the end of bioassay if compared with initial sample. It is worth noting that the same behaviour was found in ENDO and EXO sample without plastic added, while in MIX c without plastic, observed an increase after of 30 days of vermicomposting, especially in *m/z* 78 and 104, with mean signal increase of 50 %. This increased trend we assumed that is natural alteration of the functional group from evolution of organic matrix in presence of this type of microbial consortium.

After of 30 days of vermicomposting (Fig 33), for polyethylene spiked samples found a decrease in intensity of MS ions in ENDO and EXO with mean value of -21 and -22 %, respectively, which might be related to the degradation of MPs and alteration of the functional groups of plastic material (Sintim et al.,2019). The sample without PMC addition (No PMC) not showed changes if compared with Initial sample. In opposite trend, MIX c noticed an increase in signals of ions that provide information about PE, which could be related with the mass and volume loss inherent to organic matter degradation (Sáez et al.,2017). In the determination of ions related with PS, only a decrease in signal were found for No PMC treatment, whilst in EXO, MIX c and especially in ENDO (Fig 33), a higher intensity in ions measured were found. Therefore, it seems that the rate of degradation of organic matter and the loss of volume is quicker than the degradation process of PS. In agree with this finding, Sintin et al 2019 not found degradation sign in PE plastic after 18 weeks of composting. In contrast with the observed in our study, Ragoobuer et al.,2013 reported that vermicomposting contributed to the reduction (22–31%) and promoted the biodegradability of PP-MPs and HDPE-MPs. The abundance of PP HDPE, measured by FTIR technique decreased significantly by 31 % and 22 % respectively after 14 weeks of vermicomposting (measured by FTIR

technique). Based on the result obtained, we assumed that TGA-MS technique can be used to quantify the abundance of PS into organic matrix but not able to provide information about biodegradability process of this type of plastic.

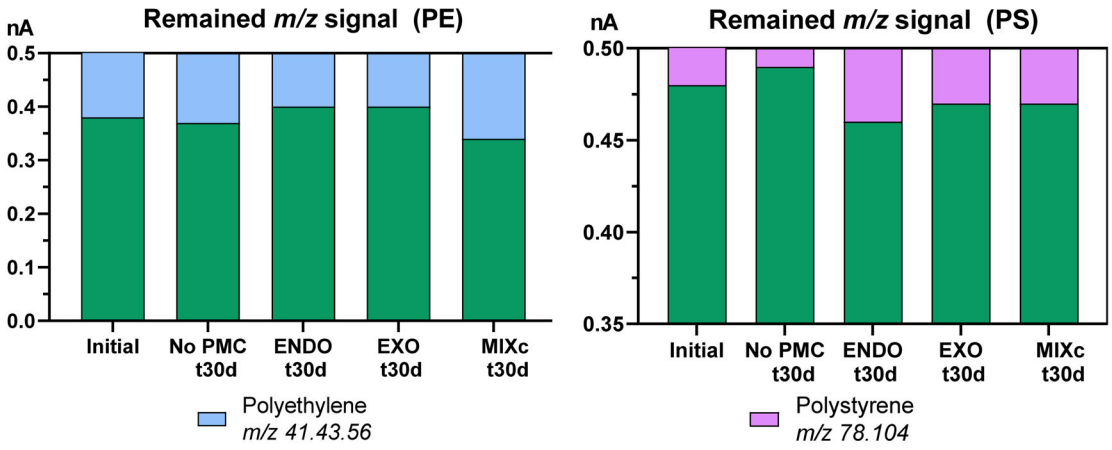


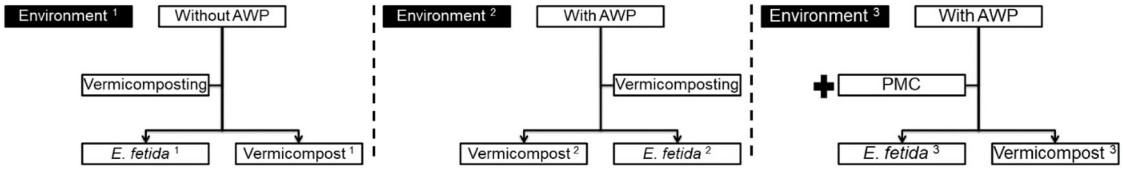
Figure 33. Relative abundance of *m/z* signal obtained in MS analysis related to PE (*m/z* 41.43.56) and PS (*m/z* 78,104).

5. Conclusions, Prospects and Challenges

5. CONCLUSIONS, PROSPECTS AND CHALLENGES

CONCLUSIONS

This thesis is focused to study how the presence of plastics in organic waste fluxes affects the vermicomposting process as valorization treatment, using *Eisenia fetida (EF)* earthworms as the main indicator and how different approaches (ageing-pretreatments, beneficial microbiome) can reduce EF affection by plastics and potentially also contribute to plastic degradation during the vermicomposting.



Related to the impact of pre-treatments approaches applied to AWP, the following statements were concluded:

- Thermochemical treatment with reagents seems to be the most effective method for the pretreatment of AWP, affecting not only the surface of the plastic probes but the whole plastic material. Time and temperature were cooperative factors for degradation.
- The effect of the UV radiation treatments depended on the exposure time and the type of plastic used, depending on whether the structures were more (PE) or less refractory (PET and PS).
- The e-beam radiation did not show a significant surface oxidation as the rest, but considering the TGA evaluation, the loss of integrity was promoted, with PS and PE standing out.

When AWP was present in vermicomposting microcosmos (Environment 2), the following statements were concluded:

- In the absence of AWP the organic substrate induced a significant increase in earthworm body weight and close to 100% survival, so the substrate used was not a limiting factor for the correct development of *E. fetida*.

- The presence of AWP developed survival losses of 10-25% in earthworms depending on the type of AWP, with polyethylene (PE) family plastic types being the most negative for EF survival. The presence of AWP also induced morphological changes in *E. fetida*, which involved losses in body mass at the end of the bioassay.
- *E. fetida* developed different molecular responses depending on the type of AWP to which they were exposed. We observed an increased detoxification response, skin damage, tissue laceration and a neurotoxic effect in *E. fetida* (highlighting LDPE, LLDPE, PET and PS).
- The vermicompost obtained in presence of AWP had worse physicochemical characteristics that the vermicompost without AWP, also producing vermicompost with lower agronomic quality (lower pH values, lower evolution of organic matter and lower nutrient content).

In the vermicomposting microcosms in the presence of AWP and inoculation with beneficial microorganisms, PMC (Environment 3), the following statements were concluded:

- Inoculation with PMC generally improved survival and decreased body weight loss of *E. fetida* at the end of the bioassay, observing different effects depending on type of PMC (EXO-PMC, ENDO or MIX-PMC) and AWP.
- Inoculation with PMC (highlighting EXO-PMC) inhibited the neurotoxic response produced by the presence of AWP in *E. fetida*. Oxidative stress (tissue damage, lacerations in *E. fetida* tissue) was inhibited with ENDO-PMC inoculation for LDPE and MIX-AWP, and with EXO-PMC and MIX-PMC inoculation for LDPE.
- Inoculation with PMC (highlighting EXO and MIX-PMC) produced a vermicompost (in the presence of AWP) more evolved, with a higher organic matter degradation. Inoculation with EXO and ENDO-PMC was able to improve the agronomic quality of the vermicompost obtained, specially increasing concentration of P and K nutrients (and maintaining N levels).
- The use of inoculum including EXO-PMC microorganisms was able to inhibit the negative biochemical effect caused by the presence of AWP in the vermicomposting process. ENDO-PMC microorganisms improved the health

status and inherent capacities of *E. fetida*, and therefore the resulted vermicompost achieved higher agronomic quality. Finally, inoculation with both types (EXO+ENDO-PMC) showed combined positive effects.

- The persistence of PMC after vermicomposting was confirmed however the degradation of AWP was negligible.

In conclusion, the obtained findings unveiled structural modifications in AWP after undergoing various pre-treatments, indicating an increased prevalence of carboxyl groups and the incorporation of more oxygen atoms into the polymer chain, being a promising way can contribute to plastic crumbling. The presence of AWP induced clear negative effects on *E. fetida*, manifested by noticeable reductions in body weight, oxidative stress and/or neurotoxicity, and even mortality in certain individuals during prolonged experiments. Furthermore, alterations were also noted in the quality and NPK content of the resulting vermicompost, particularly impacting LDPE + LLDPE, PET, and PS. The application of PMCs significantly enhanced *E. fetida* capability to mitigate the negative repercussions induced by AWP presence, leading to the production of superior quality biofertilizers.

PROSPECTS AND CHALLENGES

Sustainable waste management has become crucially important in the current context, driving research in several areas, including vermicomposting. This biological process, which involves the decomposition of organic matter by earthworms, has proven to be an effective technique for transforming organic waste into nutrient-rich biofertilizers for the soil. However, growing concerns about plastic pollution should lead several researchers to explore the implications of the presence of plastics in vermicomposting and seek solutions to mitigate these impacts. Plastic waste is ubiquitous in the environment and, by infiltrating organic waste management systems, can affect the quality of the compost produced. Plastics, being slow degrading synthetic materials, present obstacles to the efficient action of earthworms, interfering with their ability to decompose the surrounding organic matter. This raises concerns about the possible accumulation of microplastics in the final compost, which could have negative consequences for soil health, plant health and ultimately the food chain.

Prospects in this field include advances in understanding the genetic adaptations of earthworms to cope with the presence of plastics. Genomic and transcriptomic research may shed light on the molecular mechanisms that allow some species to use plastics as a carbon source. This information may be fundamental for the development of strains of microorganisms that create improved synergies with earthworms and may be able to break down plastics more effectively. The identification and characterization of enzymes responsible for the degradation of plastics are areas of great interest. Future research could also focus on finding new enzymes with improved capabilities to break down a variety of plastic polymers. Genetic engineering of microorganisms to increase the production of these enzymes could be a promising approach. On the other hand, nanotechnology could offer exciting opportunities to improve the degradation of plastics. Nanoparticles with catalytic properties could be designed to accelerate the decomposition of plastics during vermicomposting. However, further research is crucial to fully understand the associated potential environmental and safety effects. Finally, future research should also focus on developing effective methods to monitor and evaluate the biodegradation of plastics during vermicomposting. This would include chemical, genomic, and environmental analysis techniques to track the breakdown of plastics at the molecular level and assess the environmental impact over time.

In conclusion, the research on the presence of plastics in vermicomposting processes point towards a deeper understanding of the biological and genetic interactions involved.

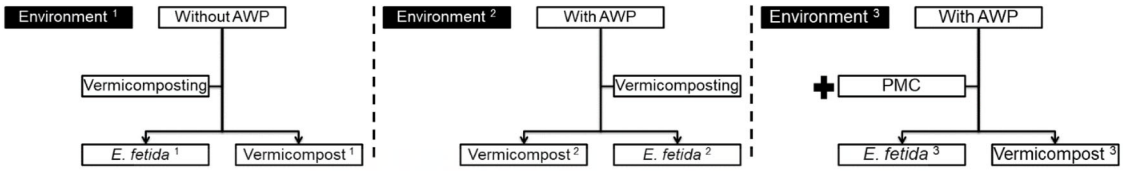
Advances in genetic engineering, biotechnology and analytical technology, could improve organic waste management, thus contributing to environmental sustainability and the preservation of soil and ecosystem health.

5.Conclusiones, perspectivas y desafíos

5. CONCLUSIONES, PERSPECTIVAS Y DESAFÍOS

CONCLUSIONES

Esta tesis se centra en estudiar cómo la presencia de plásticos en los flujos de residuos orgánicos afecta al proceso de vermicompostaje como tratamiento de valorización, utilizando lombrices de tierra de *Eisenia fetida (EF)* como principal indicado. También estudia cómo diferentes enfoques (pretratamientos de envejecimiento, microbioma beneficioso) pueden reducir la afección de FE por plásticos y potencialmente también contribuir a la degradación del plástico durante el vermicompostaje.



En relación con el impacto de los enfoques de pretratamientos aplicados a la PTA, se concluyó que:

1. El tratamiento termoquímico con reactivos químicos parece ser el método más eficaz para el pretratamiento de AWP, afectando no solo a la superficie de las muestras de plástico, sino a todo el material plástico. El tiempo y la temperatura fueron factores cooperativos para la degradación.
2. El efecto de los tratamientos con radiación UV dependió del tiempo de exposición y del tipo de plástico utilizado, dependiendo de si las estructuras eran más (PE) o menos refractarias (PET y PS).
3. La radiación E-beam no mostró una oxidación superficial significativa como el resto, pero considerando la evaluación de TGA, se promovió la pérdida de integridad, destacándose este efecto sobre PS y PE.

Cuando el AWP estuvo presente en el microcosmos de vermicompostaje (Ambiente 2), se concluye que:

1. En ausencia de AWP el sustrato orgánico indujo un aumento significativo en el peso de la lombriz y una supervivencia cercana al 100%, por lo que el sustrato utilizado no fue un factor limitante para el correcto desarrollo de *E. fetida.*

1. La presencia de AWP desarrolló pérdidas de supervivencia del 10-25% en lombrices de tierra dependiendo del tipo de AWP, siendo los tipos de plástico de la familia del polietileno (PE) los más negativos para la supervivencia de la lombriz. La presencia de AWP también indujo cambios morfológicos en *E. fetida*, lo que implicó pérdidas de masa corporal al final del bioensayo.
2. *E. fetida* desarrolló diferentes respuestas moleculares dependiendo del tipo de AWP al que estuvieron expuestos. Observamos un aumento de la respuesta de desintoxicación, daño cutáneo, laceración tisular y un efecto neurotóxico en *E. fetida* (destacando LDPE, LLDPE, PET y PS).
3. El vermicompost obtenido en presencia de AWP presentó peores características fisicoquímicas que el vermicompost sin AWP, produciendo además vermicompost con menor calidad agronómica (menores valores de pH, menor evolución de la materia orgánica y menor contenido de nutrientes).

En el microcosmos de vermicompostaje en presencia de AWP e inoculación con microorganismos benéficos, PMC (Ambiente 3), se concluyeron las siguientes afirmaciones:

1. La inoculación con PMC generalmente mejoró la supervivencia y disminuyó la pérdida de peso corporal *de E. fetida* al final del bioensayo, observándose diferentes efectos según el tipo de PMC (EXO-PMC, ENDO o MIX-PMC) y AWP.
2. La inoculación con PMC (destacando EXO-PMC) inhibió la respuesta neurotóxica producida por la presencia de AWP en *E. fetida.* El estrés oxidativo (daño tisular, laceraciones en el *tejido de E. fetida*) se inhibió con la inoculación de ENDO-PMC para LDPE y MIX-AWP, y con la inoculación de EXO-PMC y MIX-PMC para LDPE.
1. La inoculación con PMC (destacando EXO y MIX-PMC) produjo un vermicompost (en presencia de AWP) más evolucionado, con una mayor degradación de la materia orgánica. La inoculación con EXO y ENDO-PMC fue capaz de mejorar la calidad agronómica del vermicompost obtenido, especialmente aumentando la concentración de nutrientes P y K (y manteniendo los niveles de N).
2. El uso de inóculo que incluye microorganismos EXO-PMC fue capaz de inhibir el efecto bioquímico negativo causado por la presencia de AWP en el proceso

de vermicompostaje. Los microorganismos ENDO-PMC mejoraron el estado de salud y las capacidades inherentes de *E. fetida*, por lo que el vermicompost resultante logró una mayor calidad agronómica. Finalmente, la inoculación con ambos tipos (EXO+ENDO-PMC) mostró efectos positivos combinados.

3. Se confirmó la persistencia de PMC después del vermicompostaje. Sin embargo, la degradación de AWP fue insignificante.

En conclusión, los hallazgos obtenidos revelaron modificaciones estructurales en AWP después de someterse a varios pretratamientos, lo que indica una mayor prevalencia de grupos carboxilo y la incorporación de más átomos de oxígeno en la cadena polimérica, siendo una forma prometedora que puede contribuir al desmoronamiento del plástico. La presencia de AWP indujo claros efectos negativos sobre *E. fetida,* manifestados por reducciones notables en el peso corporal, estrés oxidativo y/o neurotoxicidad, e incluso mortalidad en ciertos individuos durante experimentos prolongados. Además, también se observaron alteraciones en la calidad y el contenido de NPK del vermicompost resultante, afectando particularmente a LDPE + LLDPE, PET y PS. La aplicación de PMC mejoró significativamente *la capacidad de E. fetida* para mitigar las repercusiones negativas inducidas por la presencia de AWP, lo que llevó a la producción de biofertilizantes de calidad superior.

PERSPECTIVAS Y DESAFÍOS

La gestión sostenible de los residuos ha adquirido una importancia crucial en el contexto actual, impulsando la investigación en varias áreas, incluido el vermicompostaje. Este proceso biológico, que consiste en la descomposición de la materia orgánica por parte de las lombrices de tierra, ha demostrado ser una técnica eficaz para transformar los residuos orgánicos en biofertilizantes ricos en nutrientes para el suelo. Sin embargo, la creciente preocupación por la contaminación por plásticos debería llevar a los investigadores a explorar las implicaciones de la presencia de plásticos en el vermicompostaje y buscar soluciones para mitigar estos impactos. Los residuos plásticos son omnipresentes en el medio ambiente y, al estar presentes crecientemente en los sistemas de gestión de residuos orgánicos, pueden afectar a la calidad del compost producido. Los plásticos, al ser materiales sintéticos de lenta degradación, presentan obstáculos para la acción eficiente de las lombrices de tierra, interfiriendo con su capacidad para descomponer la materia orgánica circundante. Esto plantea preocupaciones sobre la posible acumulación de microplásticos en el compost final, lo que podría tener consecuencias negativas para la salud del suelo, la salud de las plantas y, en última instancia, la cadena alimentaria.

Las perspectivas en este campo deberían incluir avances en la comprensión de las adaptaciones genéticas de las lombrices de tierra para hacer frente a la presencia de plásticos. La investigación genómica y transcriptómica puede arrojar luz sobre los mecanismos moleculares que permiten a algunas especies utilizar plásticos como fuente de carbono. Esta información puede ser fundamental para el desarrollo de cepas de microorganismos que creen sinergias con las lombrices de tierra y puedan ayudar a descomponer a los plásticos de manera más efectiva. La identificación y caracterización de las enzimas responsables de la degradación de los plásticos son áreas de gran interés. La investigación futura también podría centrarse en encontrar nuevas enzimas con capacidades mejoradas para descomponer una variedad de polímeros plásticos. La ingeniería genética de microorganismos para aumentar la producción de estas enzimas podría ser un enfoque prometedor. Por otro lado, la nanotecnología podría ofrecer interesantes oportunidades para mejorar la degradación de los plásticos. Se podrían diseñar nanopartículas con propiedades catalíticas para acelerar la descomposición de los plásticos durante el vermicompostaje. Sin embargo, es crucial seguir investigando para comprender plenamente los posibles efectos ambientales y de seguridad asociados. Por último, la investigación futura también debería centrarse en el desarrollo de métodos eficaces para controlar y evaluar la biodegradación de los plásticos durante

el vermicompostaje. Esto incluiría técnicas de análisis químico, genómico y ambiental para rastrear la descomposición de los plásticos a nivel molecular y evaluar el impacto ambiental a lo largo del tiempo.

En conclusión, la investigación sobre la presencia de plásticos en los procesos de vermicompostaje apunta hacia una comprensión más profunda de las interacciones biológicas y genéticas involucradas. Los avances en ingeniería genética, biotecnología y tecnología analítica podrían mejorar la gestión de los residuos orgánicos, contribuyendo así a la sostenibilidad ambiental y a la preservación de la salud del suelo y de los ecosistemas.

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7. Annex

7.1. Publication 1: Effect of abiotic treatments on agricultural plastic waste: efficiency of the degradation processes. Blesa Marco, Z. E., Sáez, J. A., Andreu-Rodríguez, F. J., Peñalver, R., Rodríguez, M., Eissenberg, K., Cinelli, P., Bustamante, M. A., & Moral, R. ***Polymers.*** (under review).

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Effect of abiotic treatments on agricultural plastic waste: efficiency of the degradation processes

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Abstract: In this study, four different plastic materials usually used in the agricultural sector (polystyrene film (PS), polyethylene terephthalate film (PET), low density polyethylene film (LDPE) and linear low-density polyethylene film (LLDPE)) were subjected to different abiotic treatments including photo-oxidation (ultraviolet and e-beam radiation) and thermochemical treatments to enhance polymer degradation. The degree of polymer degradation was assessed by the use of thermal and spectroscopic analyses, such as TGA and FTIR. In addition, efficiency, cost-benefits, and potential side-effects were also evaluated to propose the optimal degradation strategy to reduce plastic waste from an efficient point of view. The results obtained showed that the pre-treatments based on photo-oxidation (ultraviolet B and C and e-beam radiation) were more efficient and with a higher cost-benefit for the degradation of the polymers studied in relation to thermochemical treatments. Furthermore, the overall efficiency on the plastic degradation of the pre-treatments should be studied using a multicriteria approach, since FTIR assessment in some cases only consider oxidation processes on the plastic surface and do not show the potential integrity changes on the plastic probes.

Keywords: agri-food waste plastic; polystyrene, polyethylene terephthalate, linear low density of polyeth-ylene, low density polyethylene, thermal analyses, FTIR, e-beam.

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1. Introduction

Since 1950, plastic production has increased on an average of almost 10% every year on a global basis [1]. The great diffusion of plastic in our society is due to a range of characteristics of the plastic materials, such as their low density, lightness, strength, workability, and low cost compared to other materials. However, such diverse consumption leads to a diverse waste stream [2]. Plastic materials are widely used in European agriculture because they contribute to increase the quality and the quantity of production. About twenty distinct groups of plastics for agricultural use exist, each with various formulations available to enable the best choice for each specific application being the main polymers used in agriculture polyolefins, specifically polyethylene (PE) and polypropylene (PP) due to their low cost, good workability, high impact resistance, excellent chemical resistance and electrical insulation properties. They are mainly used to produce films (for greenhouses, low tunnels, mulching, and silage), due to its high tear and impact strength [3].

Regarding the food packaging market, European total demand for plastic has risen to 49 million tonnes per year, of which 37-38 % is used for packaging. Packaging plastics

consumed worldwide accounts for 35% [4]. Around 60% of all plastic packaging is used for food and beverages, while the rest covers non-food applications, such as healthcare, cosmetics, consumer, household, apparel, and shipment packaging being the five most common polymers used PE, PP, polystyrene (PS), terephthalate of polyethylene (PET) and polyvinyl chloride (PVC) [5]. Therefore, of the total plastic waste, 60% corresponds to packaging and 5% corresponds to agricultural plastic waste.

On the other hand, in a polymer, any physical or chemical change in its properties, is a result of environmental factors, such as light, heat, moisture, chemical conditions, or biological activity. Thus, the processes inducing changes in polymer properties (deterioration of functionality) resulting in bond scission and subsequent chemical transformations are known as polymer degradation. Considering the long degradation times for plastic waste under natural conditions, the studies are focused on the development of artificial procedures to enhance plastic degradation. These approaches may be biotic or abiotic. Thus, Mantia et al. (2017) [6] grouped the main abiotic treatments to degrade plastics in three main types:

- Thermal/thermomechanical treatments
- Photo-oxidation treatments
- Chemical/thermochemical treatments

Thermal treatments are mainly based on the action of heat; thus, the degrading effects can be very different depending on the components of the blends and on their chemical structure. Usually, degradation can be facilitated by reducing crystallinity and packaging, enhancing chain mobility and stress, but in several cases could act as stabilizers. The combination of mechanical stress with thermal variation results in a "thermomechanical" degradation. Photo-oxidation treatments are based in the degrading action of electromagnetic radiation on the polymers. The energy of the radiation, correlated to specific wavelengths, results in the break of specific chemical bonds into de polymer structure. Then, depending on the energy, different types of chemical bonds can be broken, and act as starter for degradation cascades. Chemical/thermochemical treatments are based on the action of acidic or alkaline conditions, polar or non-polar solvents and specific chemical compounds (initiators) on the integrity of polymers and blends. This kind of treatments can be very efficient on specific polymers, e.g., use of aromatic amines in PE. The combination of chemical and heat induces significant increase in degradability. Thus, the process of plastic degradation is determined by both environmental conditions and physico-chemical properties of polymeric substances. The physico-chemical properties of plastic play an important role in the degradation process specially its chemical structure as well as its hydrolysable capacity. Figure 1 shows the market shares of the most significant plastics including their hydrolysable capacity [7].

Regarding biotic approaches, the usage of microorganisms to promote degradation to a diverse group of plastics have been also evaluated [8]. Thus, techniques such as scanning electron microscopy (SEM), Fourier transform infra-red spectroscopy-attenuated total reflection (FTIR-ATR), hydrogen nuclear magnetic resonance (H-NMR), differential calorimetry scanning (DSC) and thermogravimetry coupled to mass spectrometry (TGA-MS) have been commonly applied to provide structural information during polymer degradation [9]. Other techniques, such as weight loss, C, H, and N analysis or respirometer studies (CO_2 released during the degradation process) are the most common analytical methodologies applied to assess the degradation level of PBAT-PLA due to their simplicity and low-cost [10]. However, it is scarce the number of studies that evaluate the degradation degree of several polymer of different chemical families after the application of different pre-treatments (some of them innovative such as the use of e-beam radiation), using different analytical approaches.

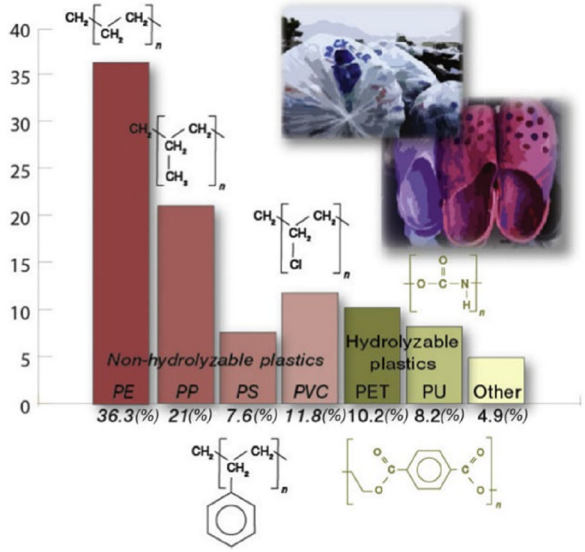


Figure 1. Market shares of the six commercially most important plastic material types [7]. Their monomer structures in high molecular weight polymer chains are shown adjacent to bars. Abbreviations: PE, polyethylene; PET, polyethylene terephthalate; PP, polypropylene; PS, polystyrene; PU, polyurethane; PVC, polyvinyl chloride.

Therefore, in this study two groups of polymers usually used in the agricultural sector and classified according to their resistance to degradation were considered: C-C backbone polymers, such as PS and PE (Low-density PE (LDPE) and Linear Low-density PE (LLDPE)), as well as an oxygen-containing polymer (PET). Selected samples of each type of polymer were subjected to different abiotic treatments including photo-oxidation, thermochemical treatments and e-beam radiation, to enhance polymer degradation. Degradation was evaluated by the use of thermal and spectroscopic analyses, such as TGA and FTIR. In addition, efficiency, cost-benefits, and potential side-effects were also evaluated to propose the optimal degradation strategy to reduce plastic waste from an efficient point of view.

2. Materials and Methods

2.1 Polymers used

Four different plastic materials were considered for this study, most of them used in agricultural activities: polystyrene film (PS), polyethylene terephthalate film (PET), low density polyethylene film (LDPE) and linear low-density polyethylene film (LLDPE), all of them in the virgin form. All these materials were provided by the University of Pisa (UNIPI) in the framework of the research project RECOVER, financed by the BBI-JU-Horizon 2020.

2.2.3. Processing conditions to enhance polymer degradation

2.2.3.1. Photo-oxidation: UV pre-treatments

The photo-oxidation treatments considered were based on the exposition of plastic films to different UV ranges and duration. Thus, the plastic probes samples (4 cm^2-20 cm^2) of PS, PET were subjected to 120 h of exposition, while LDPE and LLDPE were exposed during 750 h, at UV radiation under different wavelengths (UV-B, at 320 nm and UV-C, at 253 nm). The characteristics of the UV chambers (B and C) are shown in Table 1.

Table 1. Details of the UV chamber characteristics.

Type UV	W x chamber	Irradiated surface (m^2)	Power consumption (kW/m^2 h)
B	20	0.2479	0.161
C	16.7	0.2479	0.135

2.2.3.2. E-beam radiation pre-treatment

For the application of the e-beam radiation to the plastic materials was used an EBlab 200 device (Comet, Switzerland), specialized for low energy electron beam treatment; harbouring an EBA-200 e-beam lamp operating at accelerating voltage from 70 to 200 KeV, which correspond to a total dose energy from 100 to 1000 kGy. The analyses were carried out in ambient atmosphere. The plastic samples used were as plastic probes with maximum dimensions of 21 cm x 30 cm.

2.2.3.3. Thermochemical pre-treatments

The thermochemical pre-treatments used to favour plastic degradation were based on the exposition of plastic films to several oxidizing agents including different conditions of reaction temperature, attack duration and oxidizer concentration. Table 2 summarizes the different reagents and the conditions used for chemical degradation of the polymer samples.

Table 2. Description of the thermochemical conditions (chemical reagents/temperature/time) to enhance plastic degradation.

Treatments	Code	Plastic type	Conditions
$(NH_4)_2S_2O_8 + H_2SO_4$	AM	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	6 and 12 days, 35 and 70ºC, concentrated and diluted (100% and 33%) reagents
$K_2Cr_2O_7 + H_2SO_4$	CM	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	6 and 12 days, 35 and 70ºC, concentrated and diluted (100% and 33%) reagents
HCl : HNO_3 : H_2O 3:1:1, Aqua regia	AR	LDPE, LLDPE, LLDPE+LDPE, PP, PET, PS	6 and 12 days, 35 and 70ºC, concentrated and diluted (100% and 33%) reagents

2.3 Analytical approaches to evaluate polymer degradation

Different approaches were considered to stablish the potential damage degree of the pre-treatments on the selected plastic samples.

2.3.1 FTIR determinations

To monitor the evolution in the chemical structure of the polymer samples due to the formation or disappearance of specific functional groups infrared analysis of the samples in x mode were carried out. A spectrometer BRUKER IFS 66/S, with a resolution of 1 cm^{-1} was used. It had a source, a KBr beam splitter and two detectors: a detector DLaTGS at room temperature for measurements of routine and obtaining of spectra between 7000 and 400 cm^{-1} and other MCT detector cooled with liquid nitrogen of high sensitivity and speed of measurement, ideal for action kinetics between 600 and 4000 cm^{-1}. In addition, a JASCO FTIR 4700 spectrometer, with a resolution of 0.5 cm^{-1} was also used. It had a source of go media, a beam splitter KBr encapsulated in Germanium and a detector DLaTGS for routine measurements between 7800 and 400 cm^{-1}. FTIR spectra has been widely used to evaluate plastic degradation by determining several indexes [11-13]. These indexes are defined as the ratio between the integrated band absorbance of the functional group (or

specific bond type), which may result from polymer degradation, and that of a reference peak used to characterize the degree of oxidation of the considered polymer (eq. 1):

$$\text{Functional group index} = (\text{Absorbance functional group peak})/(\text{Absorbance reference peak}), \quad (1)$$

According to the values obtained in these indexes, the higher are the values, the greater is the degree of degradation of the polymer. Thus, the carbonyl index, defined by [11] as the absorbance of the carbonyl group at 1720 cm^{-1} versus the absorbance of the reference peak at 2920 cm^{-1} which corresponds to alkane C-H stretching vibrations of PS (Shi et al., 2019), was the main index used to evaluate polymer degradation in this study, often used as an indicator of the presence of carbonyl groups, which may result from polymer degradation [14-15]. Furthermore, additional indexes (Lactone index, Carboxyl index, Ceto Carbonyl index, Ester Carbonyl index and Hidroxyl index) [11-12;16-17] were also considered in the statistical study.

2.3.2 Thermal analysis

Thermogravimetric analysis (TGA) was performed using a Q500 thermogravimetric analyser produced by TA Instruments, under nitrogen atmosphere, on about 2 mg of sample, from room temperature to 700 °C with a heating rate of 10.00 °C/min.

2.4 Statistical analysis

The analysis of the data of the main indexes was carried out using Pareto charts, bar charts ordered by frequency counts, from highest to lowest. Pareto diagrams reflect the ordered frequency counts of values at different levels of a categorical or nominal variable, based on the 80/20 rule, which considers that 80 % of the effects are derived from 20 % of the causes. The Pareto diagrams were obtained using the IBM SPSS Statistics v. 27.0 software.

3. Results and Discussion

The main results obtained after the application of the different pre-treatments are focused on the effects on each type of plastic, considering the corresponding FTIR indexes that better explained the changes observed, as well as the efficiency, cost-benefits, and potential side-effects were also evaluated to propose the optimal degradation strategy.

3.1. Effect of the different pre-treatments on PS materials

3.1.1. Effects of the photo-oxidation pre-treatments

The photo-oxidation pre-treatments studied for polymer degradation were UV-B, UV-C and e-beam radiation, considering in the latter four different radiation levels (total dose) from 100 to 500 kGy. As it has been previously commented, FTIR indexes reflect the degree of degradation of a polymer, in this case, e.g. the higher is the carbonyl index the greater is the degree of polymer degradation since more carbonyl groups have been generated. Figure 1 shows the carbonyl index obtained from the PS polymer under the UV system type B and C under different times.

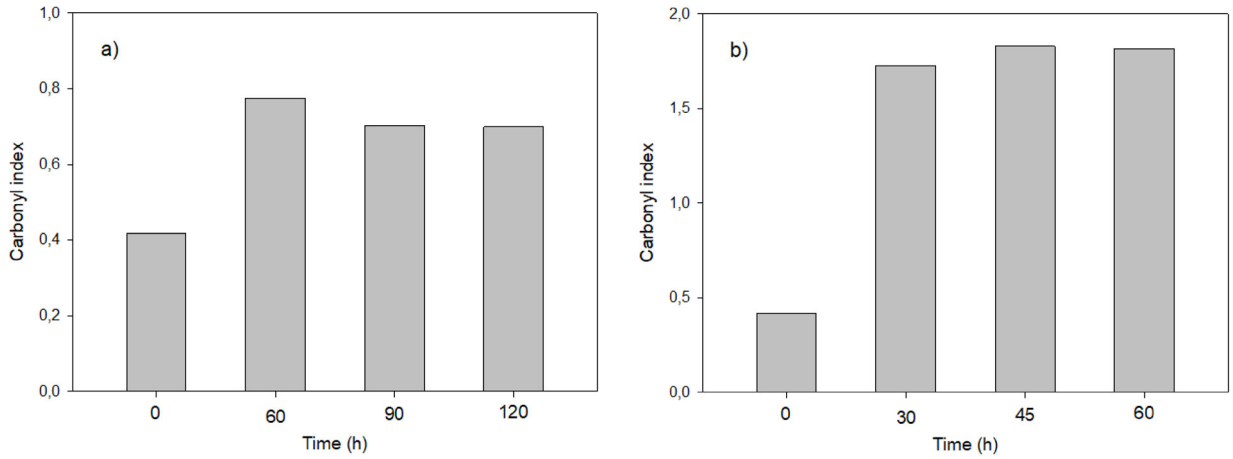


Figure 1. Values of FTIR carbonyl index used in the representative PS sample after applying during different exposure times the UV pre-treatments: a) UV-B; b) UV-C.

The pre-treatment UV-C produced the highest polymer degradation with an exposure time of 45 h, reflecting that this pre-treatment achieved the highest PS degradation compared to the application of UV-B (at any exposure time) and of the e-beam radiation, which only showed a slight increase of the carbonyl index when the total dose increased, being carbonyl indexes values lower than 0.4 in all the cases (Table 3). This could be due to the lower wavelength (low UV region) provided for the type C spectrophotometer (253 nm) than type B (320 nm). These results agree with those observed in previous works, since the presence of unsaturated double bonds in PS makes this polymer more susceptible to photo-degradation [18-19].

To validate FTIR results, TGA analysis of the PS virgin and degraded sample after the application of the photo-oxidation treatments were carried out (Figs. S1 and S2). Same conclusions were obtained by comparing the onset degradation temperature in the TGA of the different samples. Specifically, a decrease of the onset temperature of 26.6 ºC was observed when the UV-C beam was used for 30 h versus the virgin PS, this degradation pre-treatment being the most efficient. In the case of the e-beam pre-treatment, despite the carbonyl index values did not show degradation evidence, TGA results demonstrated (Fig. S3) that e-beam radiation enhanced PS degradation with a decrease in the onset temperature of almost 20ºC (residue increase of 1.5 %wt.) for an e-beam radiation of 600 kGy. Thus, although it is known that FTIR related techniques are very useful to detect surface changes, significant modifications on the entire plastic sample are better shown by TGA data. Therefore, it is important to combine different analytical techniques to get a complete evaluation of the polymer degradation processes.

Table 3. Values of FTIR carbonyl index used in the representative polymers after applying the e-beam pre-treatments.

E-beam pre-treatment	**Carbonyl index**			
	PS	LDPE	LLDPE	PET
0 kGy	0.309	0.039	0.070	0.999
100 kGy	0.277	0.063	0.087	1.002
200 kGy	0.331	0.065	0.094	1.002
500 kGy	0.373	0.097	0.125	1.004

3.1.2. Effects of the thermochemical pre-treatments

The influence of the different thermochemical conditions applied to the PS representative samples on the carbonyl index values are shown in Figs. 2a, b and c. The pre-

treatments with ammonium persulfate and chromic mixture showed the strongest oxidizing effect, since these reagents at 100% concentration seemed to produce the highest degradation in the PS sample, even observing a disintegration of at least 50% of the plastic sample at the exposure temperature of 70 ºC, independently of the exposure time (6 or 12 days). Therefore, heating is not needed if pure reagents (ammonium persulfate or chromic mixture) are used for PS degradation in 6 days at 35ºC. Thus, in general, the temperature conditions of 35ºC produced a more efficient degradation when the reactive was pure (100 %), showing in all the cases a high degradation effect. Longer degradation times (12 days) were also assessed obtaining similar results which means that no longer times than one week are needed to obtain efficient PS degradation.

Pareto charts (Fig. S2) for other FTIR indexes (Lactone index, Carboxyl index, Ceto Carbonyl index, Ester Carbonyl index and Hidroxyl index) with the aqua regia pre-treatment (for the rest of chemicals was not possible to carry out the statistical analysis due to polymer disintegration under certain conditions) were carried out to evaluate the factor (Time-A, Temperature-B or reagent concentration-C) with the highest effect on the polymer degradation. The statistical analysis shows that the concentration of the chemical reagent was the most significant factor that affects PS degradation followed by the interaction of the three factors (temperature, time and concentration).

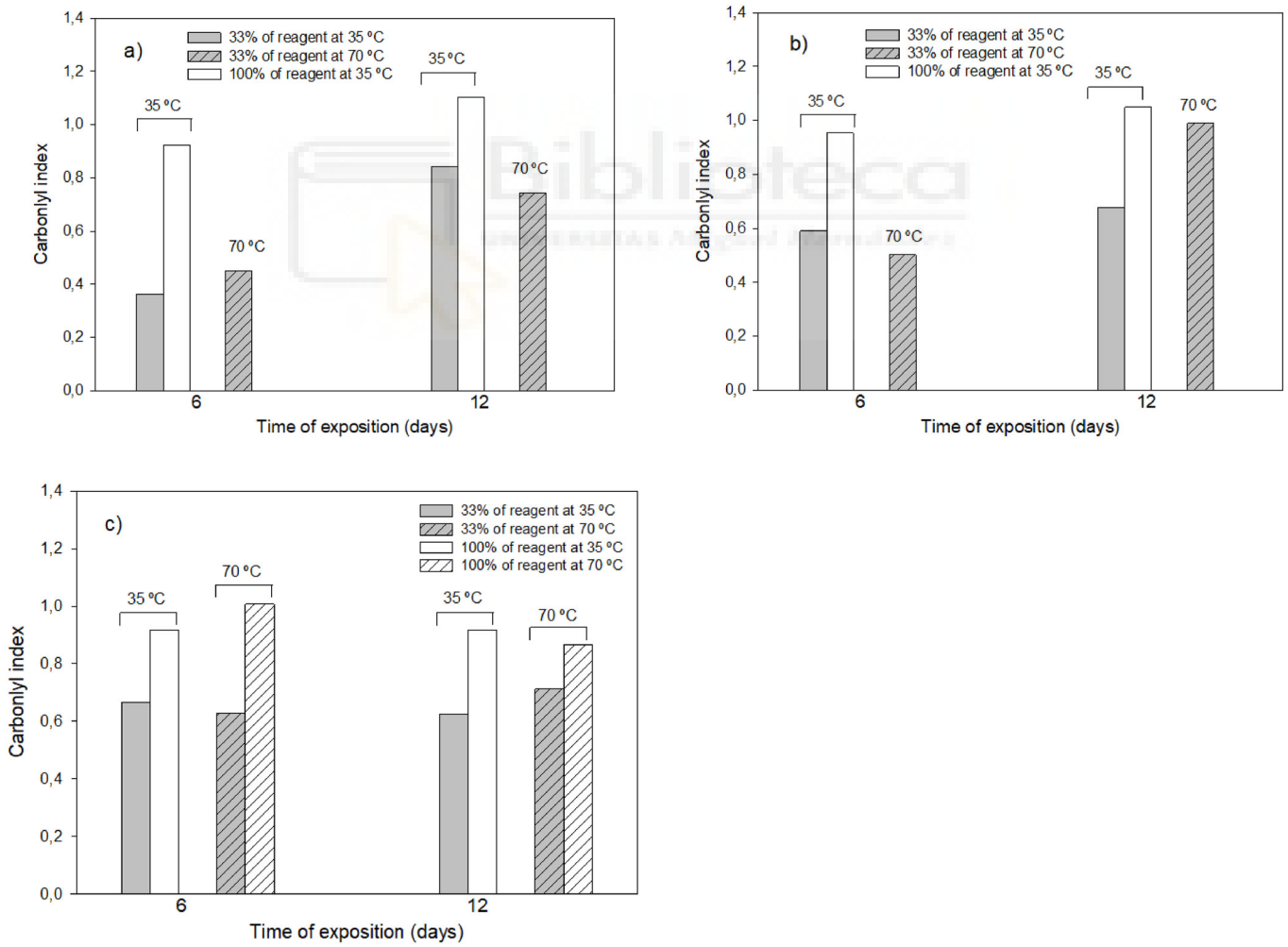


Figure 2. Values of FTIR carbonyl index used in the representative PS sample after applying during different exposure times (6 and 12 days), at different temperatures (35ºC and 70ºC) and at different reagent concentrations the thermochemical treatments: a) treatment with ammonium persulfate; b) treatment with chromic mixture; c) treatment with aqua regia. In the treatment without bar, at least 50% (dry matter basis) of plastic sample has disintegrated.

3.2. *Effect of the different pre-treatments on polyolefins (LDPE and LLDPE) materials*

Polyolefins, such as linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE), are used in many different markets due to their special properties, low production cost, light weight, and high chemical resistance. However, polyolefins are very resistant plastics with high biological and chemical inertness, whose biodegradation and natural degradation occur at very slow rates and only to a limited extent [20].

3.2.1. Effects of the photo-oxidation pre-treatments

Due to the higher resistance of the polymers LDPE and LLDPE, the exposure time considered for the UV pre-treatments and the energy applied with the pre-treatment via e-beam were higher than in the rest of plastic types previously studied. The most notable effect on the LDPE and LLDPE materials was observed for the UV-C pre-treatment at the highest exposure time (750 h) (Figs. 3a and b), which indicates that this pre-treatment produced the highest degradation in the both polymers, observing very low values of the carbonyl index for the e-beam pre-treatment (Table 3). These results indicate the high resistance of these specific polymers to photo-oxidation due to its highly stable chemical structure [21]. The chemical and physical changes occurring during the LDPE and LLDPE degradation process being correlated to the energy needed to initiate the removal of water from the bulk of the polymers. Furthermore, since LDPE and LLDPE do not contain any unsaturated double bonds, these polymers have a higher resistance to photo-degradation, but the presence of small amounts of external impurities or structural abnormalities can allow the initiation of the photo-degradation process [22-23].

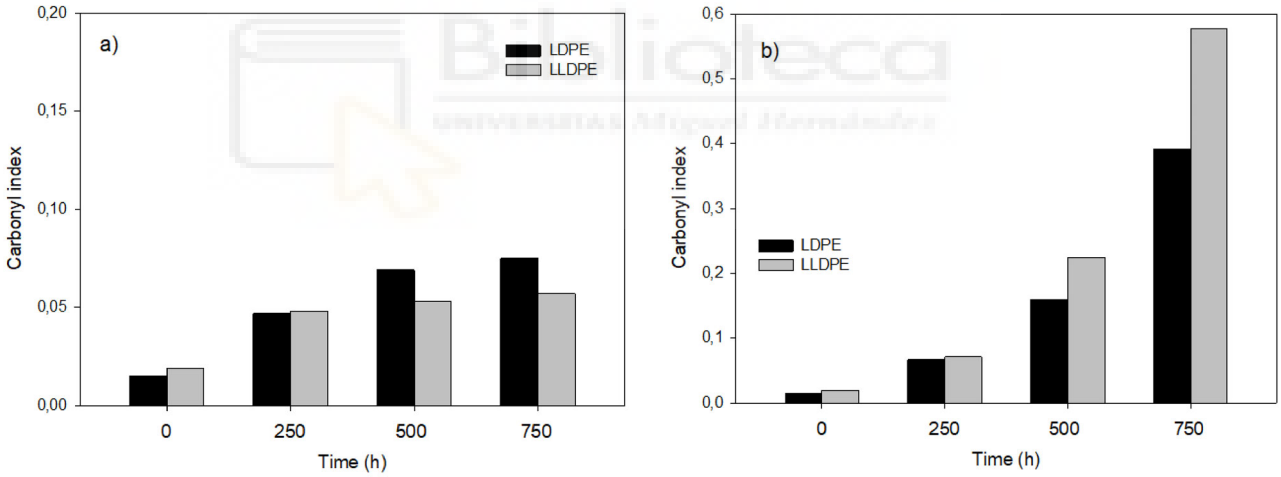
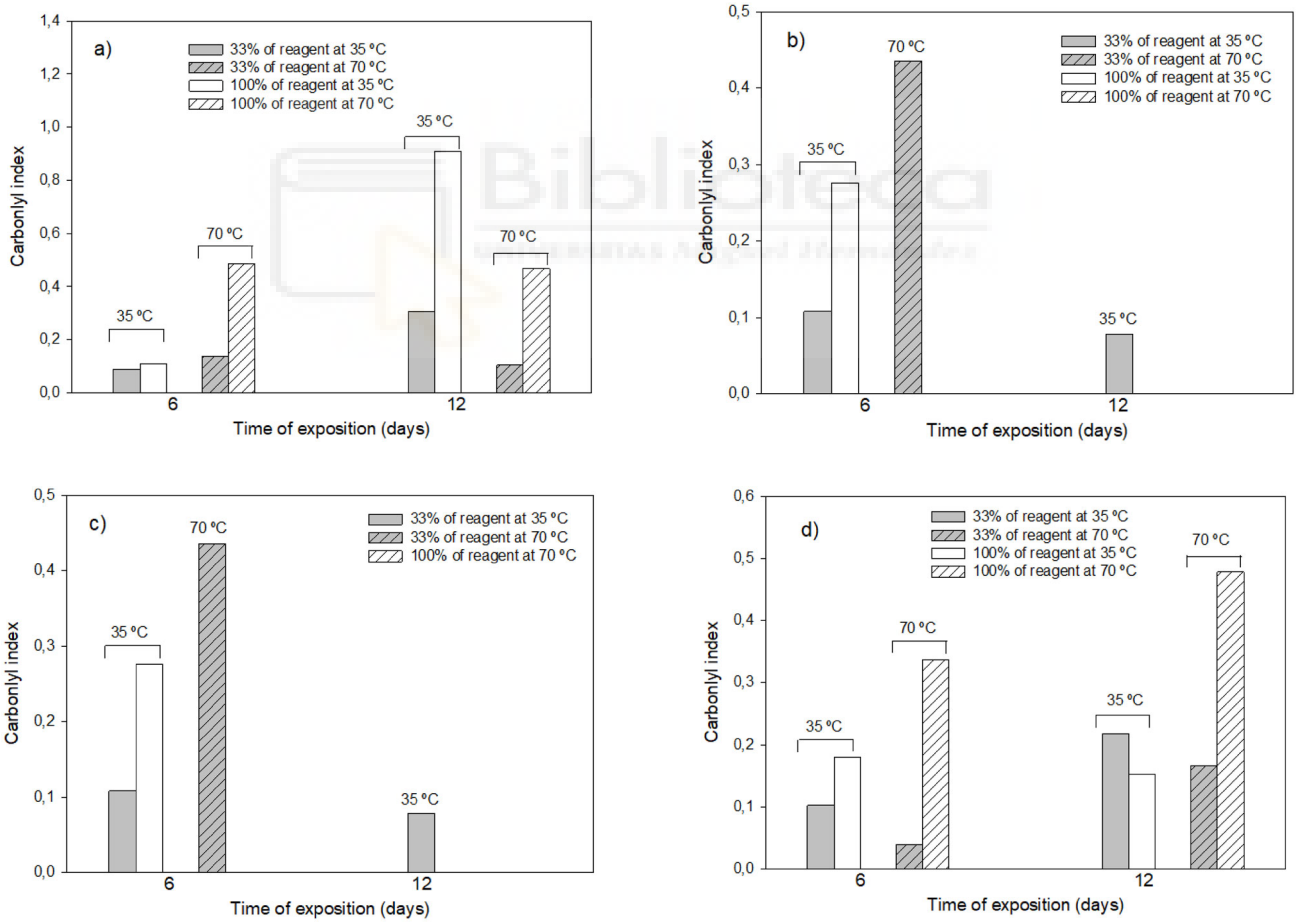


Figure 3. Values of FTIR carbonyl index used in the representative LDPE and LLDPE samples after applying during different exposure times the UV pre-treatments: a) UV-B; b) UV-C.

The effect of e-beam was not reflected in the carbonyl index values for both polymers, but TGA data showed an increase in degradation of both LDPE and LLDPE, with a decrease of the onset temperature of almost 15ºC and residue increase (1.58 %wt) of the degraded sample by e-beam (450 GKy) versus the virgin LLDPE sample. Although e-beam treatment works for both, LDPE and LLDPE, a higher effect on degradation for LDPE than LLDPE was found. It can be explained by the fact the slightly lower crystallinity of LDPE than LLDPE, which means more flexibility of the molecular chains, facilitates degradation rates. Both UV and e-beam pre-treatments affected the thermal behaviour of LDPE and LLDPE samples. Moreover, the longer treatment UV time or the higher e-beam energy the lower the LDPE and LLDPE thermal stability. However, the efficiency of the treatments was different depending on the polymer, e-beam being the more effective treatment for LDPE, and UV-C for LLDPE, as it was reported by Jeon and Kim (2014) [24] in a study of degradation of LLDPE exposed to UV-irradiation.

3.2.2. Effects of the thermochemical pre-treatments

The thermochemical pre-treatments were more efficient for LDPE degradation than photo-oxidation, unlike for LLDPE. Overall, the pre-treatments with the three oxidants at the highest concentration (pure reagent, 100%) produced the greatest degradation for both polymers, but the exposure conditions at which this effect was observed were different. Disintegration in both polymers was only observed for the chromic mixture and aqua regia pre-treatments, at the highest concentration (100 %) and temperature (70 ºC) at any exposure time (6 or 12 days) for chromic mixture, and at the highest concentration and lowest temperature (35 ºC) during 12 days for aqua regia (Figs. 4a, b and c for LDPE and 4d, e and f for LLDPE), showing that the pure chromic mixture was too aggressive, especially for the LDPE polymer, producing disintegration in most of the tests. Without considering the disintegration effect, in general, the highest degradation of LDPE was found at the lowest concentration of reagent (33%) at the exposure conditions of 70 ºC during 6 days when chromic mixture or aqua regia were used in the pre-treatment. However, for LLDPE, the best results were obtained when chromic mixture was used for 12 days at 70ºC with a concentration of 33%, since the pure reagent produced sample disintegration. The Pareto charts (Figs. S13 and S14) showed similar results in both polymers, with the exception of the aqua regia reagent for which exposure temperature was most significant for polymer degradation than chemical reagent concentration.



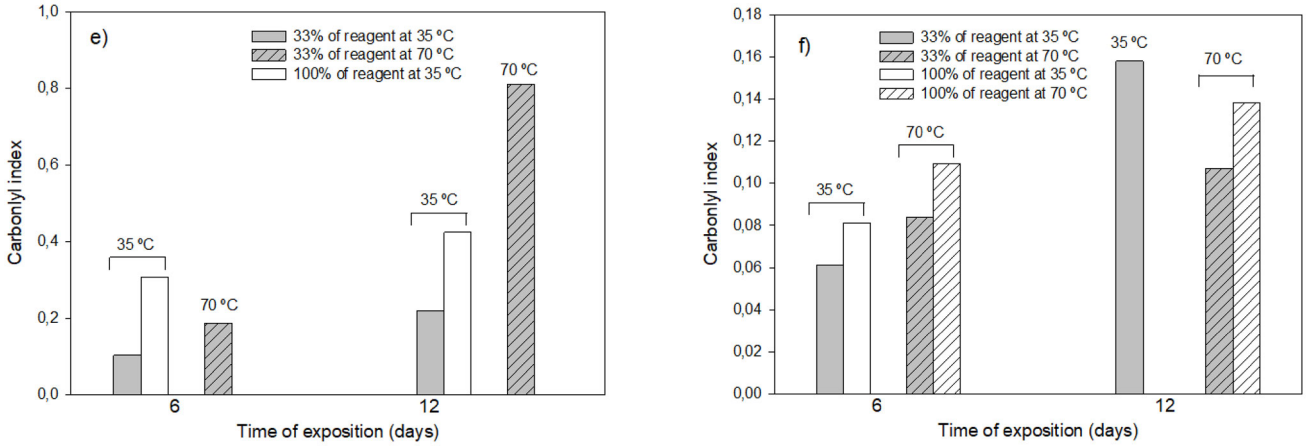


Figure 4. Values of FTIR carbonyl index used in the representative LDPE (a, b and c) and LLDPE samples (d, e and f) after applying during different exposure times (6 and 12 days), at different temperatures (35ºC and 70ºC) and at different reagent concentrations the thermochemical treatments: a) treatment of LDPE with ammonium persulfate; b) treatment of LDPE with chromic mixture; c) treatment of LDPE with aqua regia; d) treatment of LLDPE with ammonium persulfate; b) treatment of LLDPE with chromic mixture; c) treatment of LLDPE with aqua regia. In the treatments without bar, at least 50% (dry matter basis) of plastic sample has disintegrated.

The Pareto charts corresponding to the FTIR indexes of LDPE obtained after the pre-treatments with ammonium persulfate and aqua regia (the disintegration of several samples with the pre-treatment with chromic mixture made not possible to conduct the statistical analyses), showed that the concentration of the chemical reagent was the most significant factor for polymer degradation, followed by the combination of time and temperature (Figs. S11, S12, S13 and S14).

3.3. Effect of the different pre-treatments on PET materials

3.3.1. Effects of the photo-oxidation pre-treatments

The greatest carbonyl index (0.84) value was obtained with the pre-treatment using UV-B during an exposure time of 90 h (Figs. 5a and b). Therefore, further exposure times under the UV-B beam are not needed, 90 hours being the optimal exposure time to achieve maximum polymer degradation. On the other hand, UV-C beam did not produce any polymer degradation, as well as the e-beam radiation, being these treatments discarded. The presence of the carbonyl group in the polyester structure of PET facilitates photo-oxidation, indicating that PET degrades rather rapidly when it is exposed to UV light, as it has been reported in other works. Several studies have suggested that the photo-oxidation of PET involves the formation of hydroperoxide species through oxidation of the CH_2 groups adjacent to the ester linkages and the hydroperoxide species involving the formation of photo-products through several pathways [25]. Thus, the oxidation of the -CH_2 groups that produces an increase of -C=O groups in the aliphatic chains is mainly described in the 1470–1740 cm^{-1} absorption band ratio [26]. Regarding TGA results, a slight decrease of the onset temperature was observed when the UV-B beam was used for 90 h versus the virgin PET (Fig. S15 and S16). This result supports previous studies that report the strong effect of UV-B beam on PET degradation efficiency. On the other hand, TGA also showed no clear differences with the e-beam pre-treatment (Fig. S17), which pointed out the low efficiency of degradation of this procedure for PET.

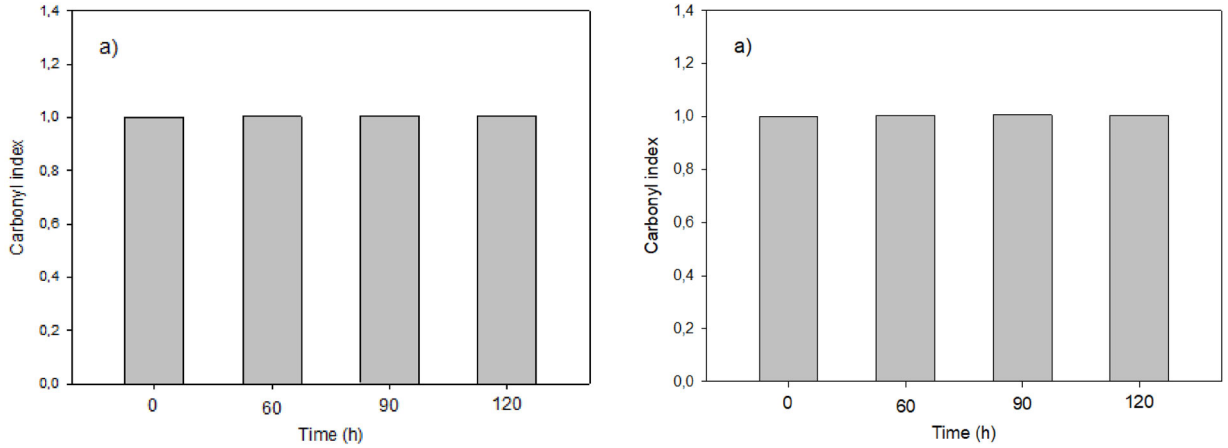


Figure 5. Values of FTIR carbonyl index used in the representative PET sample after applying during different exposure times the photo-oxidation pre-treatments: a) UV-B; b) UV-C.

3.2.2. Effects of the thermochemical pre-treatments

The degradation effect on the PET material was mainly dependent on the chemical reagent used and on the concentration of this reagent (Figs 6a and b).

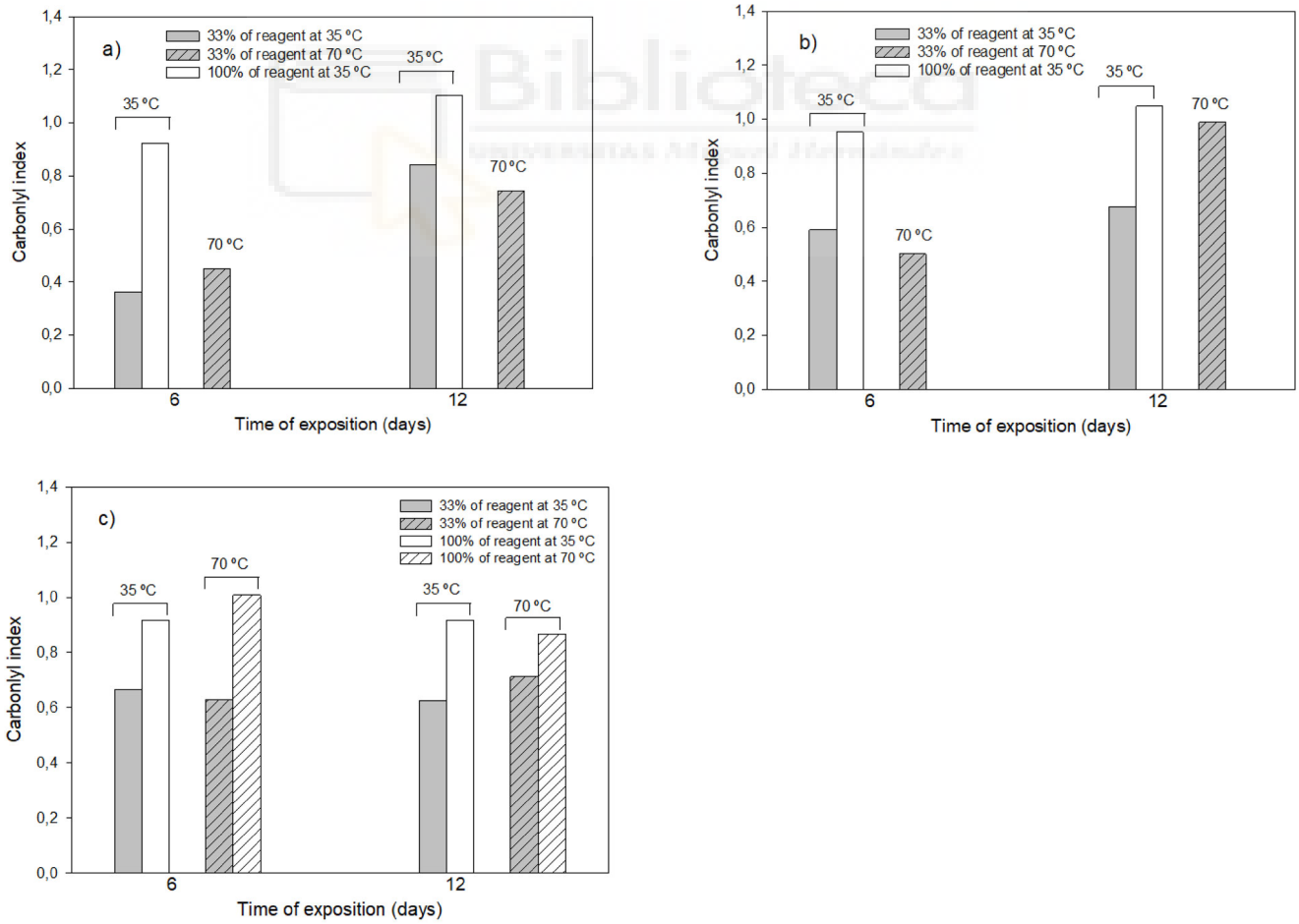


Figure 6. Values of FTIR carbonyl index used in the representative PET sample after applying during different exposure times (6 and 12 days), at different temperatures (35ºC and 70ºC) and at different reagent concentrations the thermochemical treatments: a) treatment with ammonium persulfate; b) treatment with chromic mixture; c) treatment with aqua regia. In the treatment without bar, at least 50% (dry matter basis) of plastic sample has disintegrated.

In general, the highest concentration (100%) seemed to produce the greatest polymer degradation, even with disintegration of the plastic depending on the reagent and on the exposure conditions. If the disintegration effect is not considered, in general, the greatest degradation of PET was observed at the lowest concentration of reagent (33%) at the exposure conditions of 70 ºC during 6 days with any of the chemical reagents considered. Pure chemical reagents produce disintegration of the plastic material in most of the cases, therefore it is recommended to use the diluted reagent. Thus, any of the three studied chemical reagents at 33% wt. (ammonium sulphate, chromic mixture and aqua regia) could be used for PET degradation purposes using a temperature of 70ºC for 6 days to ensure the whole polymer degradation avoiding disintegration. As all the pure oxidants produced a sample disintegration, it was not possible to carry out the Pareto charts statistical analysis.

3.3. Selection of the most efficient and cost-benefit pre-treatment procedure

The overall efficiency of used pre-treatments on the plastic degradation must be obtained using a multicriteria approach, because FTIR assessment of pre-treatments in some cases only consider oxidation processes on the plastic surface and do not show the potential integrity changes on the plastic probes. In this context, e-beam irradiation seemed to be more efficient when TGA was also used to monitor plastic modifications. To carry out a cost-effective analysis of the different pre-treatments, we have considered a qualitative classification based on three levels of costs (Table 4). The costs related to time was calculated depending on the time consumed to achieve the optimal result by each one of the pre-treatment approaches, expressed in days; the cost linked to energy consumption was expressed in energy/m^2 as a normalized approach according to our assays, considering for UV treatments the energy consumption of UV lamps, for e-beam the consumption of energy to apply the e-beam dose, both normalized by irradiated surface in the equipment. In the case of thermochemical pre-treatments, we only considered the energy consumption of the temperature control and stirring operations. The environmental cost is a complex assessment, but we have applied a simplified hierarchical approach including the binary consideration of the followed items: Greenhouse gases potential emissions (yes/no), use of water (yes/no), waste production (yes/no), energy consumption (yes/no). Each yes point as 1 and each no as a 0, being the lesser the lowest environmental cost.

Table 4. Summary of time-energy-environmental criteria established for pre-treatment cost assessment.

Time consuming (T) Costs	**Code**	**Assessment**
Low	T-L	< 24h
Medium	T-M	1-5 days
High	T-H	> 5 days
Energy consuming (E) Costs	**Code**	**Assessment**
Low	E-L	<15 kW/m^2
Medium	E-M	15-60 kW/m^2
High	E-H	> 60 kW/m^2
Environmental Cost (Env)	**Code**	**Assessment**
Low	Env-L	0-1
Medium	Env-M	2-3
High	Env-H	3-4

Table 5 summarizes the results of the cost assessment of each used pre-treatment. Time costs of UV pre-treatments varied depending on the plastic type being Medium for PS and PET and High for PE derived plastics. E-beam pre-treatments were all the lowest time consuming (<24 hours). About thermochemical pre-treatments the time cost assessment is high in all the scenarios, but additional experiments should be done to analyse lower time exposition due to the strong response of treatments, that probably can conduct

to >5 days optimal results. Similar behaviour was obtained for energy cost. Energy consumption of the UV treatments is lower for PS and PET than for polyolefins (LDPE and LLDPE). E-beam pre-treatments were all the lowest energy consuming (< 15 KW/m^2). However, chemical treatment produced a medium energy consumption for all the polymers except for PS. Environmental cost was the highest for thermochemical pre-treatments, including potential impact on GHG, water consumption, waste production and energy consumption, having UV and e-beam potential impacts of energy consumption.

Table 5. Results of cost assessment for optimal pre-treatments and plastic types.

Plastic type	UV-B	UV-C	E-beam	Thermochemical
PS				AM ≈ CM > AR
	60 h	45h	500 kGy	Concentrated>diluted
	2.5d	1,9d	<1d	12d>6d
	9.7kW	6.1kW	3.6 kW	35ºC ≥ 70ºC
				8 kW
Cost-effective assessment	T-M / E-L / Env-L	T-M / E-L / Env-L	T-L / E-L / Env-L	T-H / E-L / Env-H
PET				AM ≈ CM > AR
	90h	30h	100-200 kGy	Concentrated>diluted
	3.7d	1,3d	<1d	12d>6d
	14.5 kW	4.0 kW	2.8 kW	70ºC ≥ 35ºC
				16kW
Cost-effective assessment	T-M / E-L / Env-L	T-M / E-L / Env-L	T-L / E-L / Env-L	T-H / E-M / Env-H
LDPE				AR > CM > AM
	500-750 h	750 h	1000 kGy	Concentrated>diluted
	20-31d	31d	<1d	12d>6d
	80-120 kW	101 kW	4.9 kW	70ºC ≥ 35ºC
				16kW
Cost-effective assessment	T-H / E-H / Env-L	T-H / E-H / Env-L	T-L / E-L / Env-L	T-H / E-M / Env-H
LLDPE				CM ≈ AR > AM
	500-750 h	750 h	1000 kGy	Concentrated>diluted
	20-31d	31d	<1d	12d>6d
	80-120 kW	101 kW	4.4 kW	70ºC ≥ 35ºC
				16 kW
Cost-effective assessment	T-H / E-H / Env-L	T-H / E-H / Env-L	T-L / E-L / Env-L	T-H / E-M / Env-H

AM: ammonium persulfate; AR: aqua regia; CM: chromic mixture.

Based on these results, thermochemical treatments should be discarded due to their high time and high environmental impact for all the polymers. The opposite occurs using the e-beam treatment in which, the time, energy and environmental impact is low for all the polymers. Therefore, this new approach is recommended. As an alternative to e-beam, photo-oxidation could work very well for PS and PET in which low energy and medium

times are needed. However, for PE higher times and energy are needed to enhance degradation, therefore is not recommended for this type of polymers.

4. Conclusions

The results obtained have shown that the effect of UV radiation was plastic-dependent, observing more effective results with UV-C for PS, LDPE and LLDPE, and UV-B for PET. The suggested time of UV exposition to promote significant changes in plastics was also time-dependent, being in the range of 30-90h for PS and PET but 500-750h for PE (LDPE and LLDPE), due to its more refracting structure. Regarding the thermochemical pre-treatments. Considering all the pre-treatments studied, the thermochemical assays seemed to be the strongest approach, able to affect not only surface of the plastic probes but all the plastic material. The ammonium persulfate/chromic mixture reagents produced higher effects than aqua regia, time and temperature being cooperative factors for disintegration/degradation. E-beam radiation did not show consistent increases on FTIR indexes, indicating no so significant surface oxidation than that obtained with UV. However, considering TGA assessment, loss of integrity was promoted by e-beam considering the decrease on the temperature to achieve T98 and T95% conditions, and therefore e-beam pre-treatments must be considered especially for PS and PE plastics, PET being the least affected on these integrity parameters. Thus, the overall efficiency on the plastic degradation of the pre-treatments should be studied using a multicriteria approach, since FTIR assessment in some cases only consider oxidation processes on the plastic surface and do not show the potential integrity changes on the plastic probes. Therefore, considering the efficiency and cost-benefit of all the pre-treatments, thermochemical treatments should be discarded due to their high time and high environmental impact, being the e-beam and the photo-oxidation treatments more recommended since the time, energy and environmental impact are low for the degradation of all the polymers studied.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: TGA of the PS samples untreated (figure above) and after 60, 90 and 120 hours of UV-B treatment. Figure S2: TGA of the PS samples untreated (figure above) and after 30, 45 and 60 hours of UV-C treatment (figure below). Figure S3: TGA of the PS samples untreated and after e-beam 600 Kgy treatment. Figure S4: Pareto charts for different FTIR indexes in the pre-treatment of PS with aqua regia. Figure S5: TGA of the LDPE samples untreated and after 250 and 500 h of UV-B treatment. Figure S6: TGA of the LDPE samples untreated and after 250 and 500 h of UV-C treatment. Figure S7: TGA of the LDPE samples untreated and after e-beam treatments. Figure S8: TGA of the LLDPE samples untreated and after 250 and 500 h of UV-B treatments. Figure S9: TGA of the LLDPE samples untreated and after 250 and 500 h of UV-C treatments. Figure S10: TGA of the LLDPE samples untreated and after e-beam treatments. Figure S11: Pareto charts for the different FTIR indexes in the pre-treatment of LDPE with ammonium persulfate. Figure S12: Pareto charts for other different FTIR indexes in the pre-treatment of LDPE with aqua regia. Figure S13: Pareto charts for other different FTIR indexes in the pre-treatment of LLDPE with ammonium persulfate. Figure S14: Pareto charts for the different FTIR indexes in the pre-treatment of LLDPE with aqua regia. Figure S15: TGA of the PET samples untreated and after and after 60, 90 and 120 hours of UV-B treatment. Figure S16: TGA of the PET samples untreated and after and after 30, 45 and 60 hours of UV-C treatment. Figure S17: TGA of the PET samples untreated and after and after e-beam 300 kGy treatment.

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The Effects of Agricultural Plastic Waste on the Vermicompost Process and Health Status of *Eisenia fetida*

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Abstract: Nowadays, plastic materials are extensively used in the agri-food sector for multiple purposes. The end-of-life management of these plastics is an environmental challenge because frequent incomplete recoveries after the crop seasons lead to the accumulation of plastics debris in agricultural waste, which is now recognized as an emerging environmental issue of global concern. However, the effects of plastic debris in agricultural waste undergoing biotreatment have been poorly studied. This study assesses the effects of agricultural plastic waste (APW) (LDPE + LLDPE and EPS) (1.25% f.w.) on the vermicomposting process (45 days) in terms of earthworm health by measuring biomarker responses and the enzymatic activity and quality/stabilization of the vermicompost obtained. The results showed that exposure to all the plastic materials tested had negative morphological effects on earthworm survival and body biomass. In the vermicomposting process, the changes detected in the enzymatic activity of the vermicompost and the biofilm seemed to affect the degradation rate of earthworms and the microbiome of the substrate, as demonstrated by the low organic matter mineralization in the vermicompost exposed to plastic. Although no significant changes were recorded in several biomarkers, signs of oxidative stress were evidenced throughout the glutathione S-transferase and carboxylesterase activity, mainly involving balanced oxidative stress and xenobiotic resistance systems.

Keywords: *Eisenia fetida*; vermicomposting; ecotoxicology; earthworm; agricultural plastic waste

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1. Introduction

Different types of plastic materials widely used in agriculture have made the accumulation of plastic debris a global environmental concern. About twenty groups of plastics have been identified for agricultural use, with different formulations and a wide range of additives, such as chemicals to enhance elasticity, rigidity, UV stability, flame retardation, and color [1]. Polyethylene-based polymers are the most common plastics used in agriculture [2] because of their low cost, good workability, high impact resistance, excellent chemical resistance, and electrical insulation properties. Two grades of polyethylene plastics are low-density polyethylene (LDPE) and linear low-density polyethylene (LLPDE), which are thermoplastics made from ethylene monomers. They are mainly used to produce films (for greenhouses, low tunnels, mulching, UV protection, and silage) due to their resistance to tearing and impact [3].

Since synthetic plastics are durable because of low biodegradability, they have accumulated rapidly in the terrestrial environment, producing detrimental effects. Several studies have reported that agricultural films account for 10 to 30% of all the microplastics (MPs) accumulated in agricultural soil [4,5]. In addition to the accumulation of plastic mulch residues in farmland soils, other sources of plastic debris may be municipal waste [6], sewage sludge [7], and organic agricultural waste and its derived compost [8]. Flooding or water runoff can also lead to plastic debris accumulation in water bodies [9]. Little is known about the specific amount of plastic waste found in bio-waste, but in a study on the sewage sludge produced in a wastewater plant in Europe, the sludge contained between 1000 and 24,000 mg kg^{-1} of plastic debris [10].

Composting is an environmentally friendly and organic method of bio-waste management. However, the safe use of compost or vermicompost must be guaranteed. For this, a complete characterization of these materials must be carried out prior to their use by the determination of the main chemical parameters commonly used to evaluate the maturity and stability reached in the organic matter content, e.g., water soluble carbon or humic and fulvic acid compounds [11] or even biological parameters, such as enzymatic activities, which are the most suitable methods to assess changes in aerobic biological activities [12]. Historically, composting has been used to recycle agricultural waste, as well as many other organic wastes, and the composted organic matter is returned to the soil to maintain soil fertility and crop productivity with minimal synthetic chemical fertilizer use.

However, due to the accumulation of plastic debris in bio-waste, one of the main challenges facing the compost industry today is contamination with plastic waste. Although most of this plastic can be removed before and after composting by sieving and manual sorting, and biodegradable plastic might be degraded during composting, plastic is still commonly found in the final product. In previous studies [1,13], concentrations of visible plastic ranging from 2.38 mg to 1200 mg kg^{-1} of compost were found in different types of compost from commercial composting plants. Consequently, compost must be seen as a serious entry route for plastic in soil. Such plastic inputs may be especially problematic in agricultural soil. A yearly application of 7 t ha^{-1} to 35 t ha^{-1} can lead to an annual plastic input of between 1.2 and 6.3 kg ha^{-1} to arable fields.

Several authors have investigated the combined use of composting and vermicomposting to treat different organic materials, showing that prior composting can accelerate degradation and improve the stabilization of the final product [14–16]. In addition, the use of earthworms allows for the assessment of the potential hazardous characteristics of bio-waste, evaluating their ecotoxicological responses by analyzing several oxidative stress biomarkers (catalase, glutathione S-transferase, and thiobarbituric acid reactive substances), which are involved in antioxidant enzymatic activity changes and in the production of several molecules related to responses of their immunological system [17].

This added-value fertilizer in vermicomposting is often reflected in the price. In Spain, the mean value of commercial compost is 34 € t^{-1}, while vermicompost can reach 200 € t^{-1}. Therefore, vermicomposting represents a cost-effective biotreatment with low technical requirements to convert organic waste into stabilized humic-like products.

The effect of accumulated plastic waste has been widely studied in marine [18–21] and terrestrial ecosystems [22–24].

Plastics in soil adversely affect plant health and soil fertility [25], water holding capacity, and soil microbial activity [26]. Microplastics may also act as carriers of other pollutants such as heavy metals, increasing their bio-accessibility [27]. Furthermore, in a study on plastic material added to soil, the authors in [28] reported that plastic material might act as a microhabitat, being rapidly colonized by microorganisms that form a dense biofilm on the surface of the plastic, named the plastisphere. This plastisphere consists of several layers. In the closest layer to the plastic surface, known as the ecological corona, the reactivity of the plastic material is higher, and partial inhibition of microbial activity can be observed. Biofilm formation on non-compostable plastics, such as LDPE, polystyrene (PS), and polyethylene terephthalate (PET), has also been described in marine ecosystems [29].

However, no study has explored the formation of biofilm on plastics exposed to organic matrix substrates such as compost or vermicompost. These could act as potential biostimulants due to their inherent microbiome content and activity. Therefore, despite the growing concern about plastic accumulation in bio-waste from agro-industrial activities, little is known about the effect of plastics in bio-treatment processes, such as composting or vermicomposting, their induced effect on enzymatic and hydrolytic activity, or the agronomical quality of the bio-fertilizer obtained.

The main aim of this study is to assess the effect of the presence of different plastics used in agriculture (LDPE + LLDPE and EPS) on the vermicomposting of bio-waste at a lab scale. The following implications were studied: (1) the evolution of the vermicomposting process and quality and stabilization of the final vermicompost; (2) the response and health of *E. fetida* (EF) by measuring the main biomarkers related to oxidative and damage stress; and (3) the enzyme activity in vermicompost and plastic-biofilm provoked by earthworms interacting with plastic material.

2. Materials and Methods

2.1. Experimental Design

The experimental design consisted of a lab-scale bioassay where earthworms were exposed to different types of agricultural plastic waste (APW) in bio-waste under vermicomposting conditions. Three plastic materials commonly used in agriculture were selected: low-density polyethylene (LDPE) + linear low-density polyethylene (LLDPE) black film, LDPE + LLDPE perforated film, and expanded polystyrene (EPS). To determine the normal behavior of *E. fetida* in feedstock, three replicates (n = 3) were prepared as control treatments with only feedstock and earthworms and no plastic material added, three replicates with feedstock with plastic added and no earthworms as composting treatment, and three replicates with feedstock, plastic added, and earthworms as vermicomposting as treatment for each type of plastic material tested. The bioassay consisted of an incubation (45 days) period in Petri dishes (15 cm ø) with 80 g of feedstock adjusted with distilled water to 70% moisture content. Then, 1 g of plastic material was added per replicate (1.25% f.w. proportion) and inoculated with 25 citellated *E. fetida* adults to simulate the vermicomposting treatment. The incubation containers were kept in isolated chambers under controlled conditions (20 ± 2 °C and darkness).

The dose of plastic material (1.25% f.w.) added to the compost for this study was selected following the guidelines of Regulation (EU) 2019/1009 of The European Council on the limit of impurities (2 mm) of glass, metal, or plastic in commercial compost. Therefore, to consider the remaining material as compost fertilizer, the APW in the microcosm assays was below this range.

2.2. Experimental Set-Up

2.2.1. Feedstock Characteristics

In the exposure bioassay, compost made from agroindustrial waste was used as feedstock. This compost was developed in the COMPOLAB-UMH facility at a commercial-pile scale (10 m^3) using three ingredients (agri-food sludge + cow manure + vineyard pruning, in proportions of 45 + 15 + 40 vol %, respectively). The initial mixture was done with these proportions in order to adjust the C/N ratio to values close to 25 to improve the development of the composting process. The composting process lasted 96 days, including four turning events. The composting was used as pre-treatment to remove compounds harmful to earthworms, such as ammonium, and was stopped when the material completed the thermophilic phase. High-quality standards were achieved in terms of stabilization, sanitization, and the absence of phytotoxic effects (Table 1). The heavy metals also complied with fertilizer regulations (Regulation (EU) 2019/1009).

Table 1. Characteristics of the feedstock used.

Physicochemical Parameters					Macronutrients				Mature Parameters		
pH	**EC (dS m^{-1})**	**BD (g L^{-1})**	**TOM (%)**	**TOC (g kg^{-1})**	**TN (%)**	**P (%)**	**K (%)**	**GI (%)**	**C_{HA} (%)**	**C_{FA} (%)**	**CEC (meq 100 g^{-1} MO)**
7.8	4.5	486	63.0	225	2.13	0.41	1.01	108	1.93	2.27	128

EC: Electrical conductivity, BD: Bulk density, OM: Organic matter, TOC: Total organic carbon, TN: Total nitrogen, GI: Germination index, C_{HA}: Acid humic-like carbon, C_{FA}: Acid fulvic-like carbon, CEC: Cation exchange capacity.

2.2.2. Earthworms

The earthworms used in this study were obtained from large rearing containers (0.5 m^3) kept under controlled conditions (20 ± 2 °C and darkness). The earthworms were fed on the same feedstock used in the lab bioassay for 30 days to improve their adaptability. Adult citellated earthworms with a body mass of between 250 mg and 600 mg were selected, as recommended by international guidelines [30]. When mortality was observed during exposure, the worms were immediately removed from the rearing container.

2.2.3. Plastic Material

Three different plastic materials commonly used in agriculture were tested. Two kinds of plastic film (black film and perforated film) purchased from SolPlast Company (Murcia, Spain) composed by a mixture of low-density polyethylene (LDPE) and linear low-density polyethylene (LLDPE), which are commonly used in mulching film, were used. The LDPE + LLDPE resins have better mechanical properties than LDPE, such as higher tensile strength and impact and puncture resistance. These features make them more resistant to biodegradation. We also tested expanded polystyrene (EPS), which is used in pots for seedlings in horticultural crops. Small, irregular-shaped pieces of approx. 1 cm^2 were cut with scissors. The EPS materials were cut into small pieces of approx. 1 cm^2 and 1–2 mm thick with a steel guillotine.

2.2.4. Biofilm Sample

As previously mentioned, plastic debris acts as a habitat, and it is colonized by microorganisms that form a dense biofilm on the plastic surface. To determine the behavior of the enzymatic activity in the biological corona of the biofilm, we took samples of this by carefully separating small pieces of plastic from the vermicompost/compost and scraping them with a spatula until the plastic pieces were clean, and the substrate attached was collected and treated the same as vermicompost/compost samples for the posterior enzymatic measures.

2.3. Analytical Methods

2.3.1. Vermicompost Physicochemical Parameters

After homogenization, each vermicompost sample was divided into two subsamples. One was used immediately to determine the moisture content, and the other subsample was frozen at −80 °C to monitor the enzyme activity, and the other was kept at 45 °C in an oven with forced aeration to dry. This subsample was then ground to obtain dust particles using an agate ball mill (RESTCH mod. MM400). The particles were then left to dry at 105 °C to further analyze physicochemical parameters.

The physicochemical parameters in the vermicompost samples were analyzed as follows: electrical conductivity (EC) and pH were measured in a 1:10 water extract (*w/v*); moisture content was determined after drying to a constant weight at 105 °C for 24 h; total organic matter (TOM) content was measured by loss on ignition at 430 °C for 24 h; and total organic carbon (TOC) and total nitrogen (TN) were determined by burning the samples at 1020 °C in an automatic elemental micro-analyzer (EuroVector Elemental Analyzer, Milano, Italy). After digestion (HNO_3/H_2O) (1:1, *v/v*) of dry samples in the microwave system (CEM, mod. MARS ONE), macronutrients such as P and K, among others (Ca, Cu, Mg, Fe, Mn, Zn), and toxic heavy metals (Cr, Ni, Cd, Hg, Pb) were measured by ICP-OES.

The humic-like content was measured in an extract with 0.1 M NaOH, from which fulvic acid-like C (C_{FA}) was separated through acid precipitation of the humic acid-like C (C_{HA}). The extracted (C_{EXT}) and supernatant (CFA) were analyzed in an automatic carbon analyzer for liquid samples (TOC-V CSN Analyzer, Shimadzu Company, Kyoto, Japan). The water-soluble carbon (WSC) was measured in a 1:20 water extract (*w/v*) using the same automatic analyzer for liquid samples.

2.3.2. Vermicompost and Biofilm Enzymatic Activity

The vermicompost samples were homogenized by grinding the aggregates in a ceramic mortar and adding H_2O. The water suspension was at a ratio of 1:50 (*w/v*), namely, 1 g to 50 mL H_2O. Suspensions were carried out at the moment of preparation or maintained at 4–5 °C for a maximum of 3 days. The biofilm was carefully separated from the substrate and scraped with a spatula until the plastic was clean. Later, the sample was homogenized at a ratio of 1:10 (*w/v*), namely, 0.1 g to 10 mL H_2O.

Carboxylesterase activity (CbE) (EC 3.1.1.1.) was measured by pouring aliquots (100 µL) from the sample and adding 380 µL of Tris-HCl 0.1 M buffer (pH 7.0). The enzymatic reaction was initiated by adding 20 µL of 1-naphthyl butyrate substrate (1-NB) (2 mM, final concentration) and waiting 5 min before stopping the reaction. The formed product (1-naphthol) was revealed by adding 50 µL of Fast Red ITR salt to 0.1% (*w/v*), dissolved at 2.5% (*w/v*), and Triton X-100 at 2.5% (*v/v*). Finally, the absorbance of the naphthol-Fast ITR complex was measured at 450 nm using an Asys HiTech UVM340 microplate reader (Asys HiTech Gmbh, Eugendorf, Austria). Carboxylesterase activity was expressed as nmol h^{-1} g^{-1} of dried substrate, determined by a calibration curve built for 1-naphthol. Control (without substrate) and blank samples (without vermicompost) were used to correct the background absorbance and non-enzymatic hydrolysis of the substrates, respectively.

Dehydrogenase (DHE) activity was measured by weighing 0.1 g of sample and adding 750 µL of Tris-HCl 0.1 M buffer (pH 7.0) + 1 mL INT. This was homogenized in a vortex and kept at 40 °C in a water bath for 1 h in darkness (samples were shaken every 20 min). The reaction was stopped by adding 2.5 mL of stop solution prepared as a mixture of N-N′ dimethyl and ethanol in a 1:1 (*v:v*) relation. Two random controls were prepared with 750 µL TRIS without INT. The plate spectrometer measurements were read at 450 nm.

To determine the catalase (CAT) (EC 1.11.1.6.) activity, 1 mL was collected from the 1:50 (*w/v*) aqueous suspension and dispensed with 125 µL of hydrogen peroxide (H_2O_2). It was put in a rotor for 10 min to allow the reaction to take place and was then stopped with 125µL 3 M of sulfuric acid.

2.3.3. *Eisenia fetida* Survival and Body Weight

After 7, 21, 30, and 45 days of the exposure assay, the earthworms were gently extracted from the feedstock of each replicate (Petri dish) by hand. Then they were counted for survival, weighed in a precision scale, and this information was recorded. At the end of the microcosm bioassay (45 d), the worms in each replicate were sampled.

2.3.4. Earthworm Biomarkers

Six earthworms randomly selected from each test replicate were used for this analysis; the selected earthworms were previously depurated (24 h) in order to eliminate the organic substrate of the gut tract. The earthworms' bodies were homogenized in ice-cold buffer (pH = 7.4) made of 25 mM sucrose, 20 mM Tris-HCl buffer, and 1 mM EDTA by milling with potter (Heidolph Company). The homogenates were centrifuged at 9000× *g* for 20 min at 4 °C to obtain the post-mitochondrial fraction, which was aliquoted and stored at −80 °C until analysis.

The total protein content of *E. fetida* was determined in a 1:10 (*v:v*) aqueous dilution with bicichoninic acid (BCA). The reagent was heated at 60 °C for 15 min, and then read on the spectrometer at 630 nm. Acetylcholinesterase (EC 3.1.1.7) activity was spectrophotometrically determined in the presence of 3 mM acetylthiocholine iodide as substrate and 0.1 mM of DTNB (5.5′-dithiobis-2-dinitrobenzoic acid) by measuring the increased absorbance during the kinetic reaction, read at 412 nm. The enzymatic reaction rate was quantified against a blank without substrate for each measurement. To subtract the spontaneous hydrolysis of the substrate, a second blank was performed without the sample. Acetylcholinesterase was expressed as nmol min^{-1} mg^{-1} protein.

To determine CbE, 100 µL of homogenized tissue was added to 380 µL 0.1 M Tris-HCl buffer (pH = 8.4) and 40 µL 1-naphthyl butyrate (1-NB) 20 mM. The tubes were incubated at 20 °C for 10 min and then centrifuged for 5 min at 10,000 rpm. Then 150 µL of supernatant was transferred to new microplates, and the formation of 1-naphthol was revealed after adding 50 µL of a solution containing 0.1% Fast Red ITR. The microplates were stored in darkness for 20 min, and then the absorbance of the naphthol–Fast Red ITR complex was read at 450 nm.

Lipid peroxidation was measured in 50 µL of homogenized tissue added to 450 µL of reactive acid 2-thiobarbituric (TBAR) and butylhydroxytoluene (BHT). The reaction was maintained for 30 min at 90 °C. After that, 250 µL was dispensed in 96 deep-well microplates and read at 492 nm in a spectrometer. Enzyme activity was expressed as µg MDA mg protein. Three randomized samples were carried out without TBAR.

The glutathione-dependent antioxidant enzymes glutathione reductase (GR) (EC 1.6.4.2) and glutathione S-transferase (GST) were measured using the method described by [31,32], respectively. GR activity was determined in an aliquot of 50 µL of homogenized *E. fetida* body tissue in a reaction medium of 100 mM Na-phosphate buffer adjusted to pH 7.5, 1 mM oxidized glutathione (GSSG), and 60 µM NADPH. The kinetic reaction was measured at 340 nm in the spectrophotometer to determine the rate of NADPH oxidation. Specific enzyme activity was calculated using the extinction coefficient of 6.22 M^{-1} cm^{-1}. Glutathione S-transferase was measured in a reaction mixture containing 100 mM Na-phosphate buffer adjusted to pH 6.5, 2 mM CDNB (1-chloro-2,4-dinitrobenzene), 5 mM reduced glutathione (GSH), and 30 µL of sample. The extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to express the specific enzyme activity.

2.3.5. Statistical Analysis

The IBM SPSS Statics V.28 software package was used for the statistical analyses. To assess the significant differences in the results measuring survival, weight variation, exoenzyme activity in the vermicompost, and biomarkers, the multivariate general linear model (GLM) was used, considering the effect of t main variables (EF presence, plastic format, APW presence). LSD tests were also conducted with Tukey-b and DMS as post hoc tests.

We used factorial analysis of variance to determine the statistically-significant differences between EF presence, type of APW, and the interaction between these two factors. When the differences were significant, one-way analysis of variance (ANOVA) and the least significant difference (LSD) were conducted to establish the significant differences between means. Normal distribution and variance homogeneity were checked using the Shapiro–Wilk and Levene tests, respectively, before ANOVA.

3. Results

3.1. Effect of AWP on Vermicompost Physicochemical Parameters

The pH values at the end of the bioassay remained in a suitable range for earthworm and microorganism activity (5.5–8.5) [33] in all the treatments. As shown in Table 2, significant differences were observed in the vermicomposting process with earthworms and without earthworms. In general, the pH values significantly increased in containers without earthworms and with plastic materials. The EC values in the vermicompost also showed significant differences. In the presence of APW material, the samples with earthworms showed the highest final EC values compared to the samples without earthworms, regardless of the type of plastic material tested. No significant difference was found in organic matter content with the presence of earthworms. All the treatments showed a decrease in total organic matter at the end of the bioassay compared to the initial feedstock. Slight differences were found among the plastic materials tested. The vermicompost samples exposed to EPS had the highest values of TOM at the end of the bioassay. TOC content showed a general sharp decrease in all the treatments when compared to the initial feedstock. Nitrogen increased significantly in all the treatments, regardless of the type of plastic material. As can be seen in Table 2, when comparing the results by types of plastic, the vermicompost sample with earthworms had the lowest TN values. The WSC values showed significant differences for both *Eisenia fetida* presence and type of APW plastic. The sample with *E. fetida* presence had lower values of WSC than that without the earthworm (Table 2). The concentration of humic acid compounds at the end of the bioassay showed a slight significant difference when comparing the treatments with earthworms and those without earthworms. Additionally, the results did not show any differences between the plastic treatments and the control treatment, except for LDPE + LLDPE black film, whose samples, both with earthworms and without earthworms, had the highest values of humic acid compounds.

Table 2. Evolution of the main physicochemical characteristics of compost/vermicompost.

E. fetida Presence	Type of APW	pH	EC (dS m^{-1})	TOM (%)	TOC (%)	TN (%)	P (%)	K (%)	WSC (g kg^{-1})	C_{FA} (%)	C_{HA} (%)
Yes	No plastic t = 45 d	7.42 a	5.42	52.9	23.7 b	2.24 a	0.81	1.28	8.83 a	3.66 c	3.58
	LDPE + LLDPE black film	7.24 a	4.81	53.6	26.2 c	2.31 ab	0.82	1.27	9.00 a	2.63 a	4.11
	LDPE + LLDPE perforated film	7.46 a	4.42	55.7	24.4 b	2.29 ab	0.82	1.16	8.36 a	2.47 a	3.27
	EPS seedling	7.21 a	4.56	57.1	24.6 b	2.24 a	0.81	1.19	8.33 a	2.45 a	3.17
No	No plastic t = 45 days	7.88 b	4.17	55.3	27.3 c	2.53 c	0.72	1.36	9.89 b	2.57 a	3.16
	LDPE + LLDPE black film	8.00 bc	3.26	52.4	24.3 b	2.38 b	0.83	1.08	10.3 b	3.31 b	4.96
	LDPE + LLDPE perforated film	8.20 c	3.20	54.4	24.0 b	2.34 b	0.81	1.12	9.23 b	3.01 b	3.76
	EPS seedling	8.20 c	2.90	55.2	22.6 a	2.25 a	0.81	1.11	10.2 b	2.71 ab	3.87
Main effects											
E. fetida presence	Yes	7.35 a	4.92 b	54.4	24.8 b	2.26 a	0.81	1.22	8.41 a	3.01 b	3.52 a
	No	8.07 b	3.38 a	54.3	24.4 a	2.37 b	0.79	1.19	9.82 b	2.86 a	3.89 b
Type of APW	No plastic t = 45 d	7.57 a	5.0 c	53.7 ab	24.9 b	2.33 b	0.78	1.28	8.83	3.32 c	3.42 a
	LDPE + LLDPE black film	7.61 a	4.04 b	53.0 a	25.3 b	2.34 b	0.82	1.18	9.65	2.97 b	4.53 b
	LDPE + LLDPE perforated film	7.83 c	3.81 a	55.0 ab	24.2 a	2.31 b	0.81	1.14	8.80	2.74 a	3.52 a
	EPS seedling	7.70 b	3.73 a	56.1 b	23.6 a	2.24 a	0.81	1.16	9.26	2.58 a	3.58 a

Table 2. *Cont.*

E. fetida Presence	Type of APW	pH	EC (dS m^{-1})	TOM (%)	TOC (%)	TN (%)	P (%)	K (%)	WSC (g kg^{-1})	C_{FA} (%)	C_{HA} (%)
Statistical significance											
	E. fetida presence	***	***	ns	***	**	ns	ns	***	***	*
	Type of APW	***	***	*	***	***	ns	ns	ns	***	**
	E. fetida x APW	***	ns	ns	**	***	ns	ns	***	***	ns

EC: Electrical conductivity, TOC: Total organic carbon, OM: Organic matter, Cw: Carbon water-soluble. ns, *, **, *** indicate not significant, statically significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively. Average values ($n = 3$) in a column followed by the same letter are not significantly different at $p < 0.05$ (Tukeys and DMS test).

Regarding the heavy metal content in the vermicompost, although concentrations of Cu, Zn, Cd, and Co slightly increased after the vermicomposting process, probably due to the organic matter degradation and subsequent reduce of volume, the final levels met the European Union Eco-label requirements for ecological production. Therefore, they can be used as organic amendments in agriculture (Regulation (EU) 2019/1009). The concentrations of Cr and Ni were below detection limits (<0.01 mg kg^{-1}).

Dehydrogenase activity (DHE) is commonly used to measure overall microbial activity, since it is involved in the respiration chain of all microorganisms [34]. The DHE/WSC ratio links microbial activity with the amount of easily metabolized organic matter. In our study, the initial feedstock showed a high amount of WSC (18.9 g/kg), which could lead to a quick increase in degradative and hydrolytic activity by the microorganisms in feedstock and the gut microbiome of earthworms (Figure 1). In all the treatments, the vermicompost WSC decreased at the end of the bioassay, suggesting substrate depletion and indicating the correct evolution of microbial activity and the biotransformation of the available organic matter into more stable molecules. The DHE/WSC ratio showed remarkable differences depending on the factors used in the statistical analysis, namely, type of plastic and earthworm presence. The LLDPE + LDPE black film and LLDPE + LDPE perforated film without earthworms had the highest values for that parameter (8.06 and 8.00, respectively) (Figure 1).

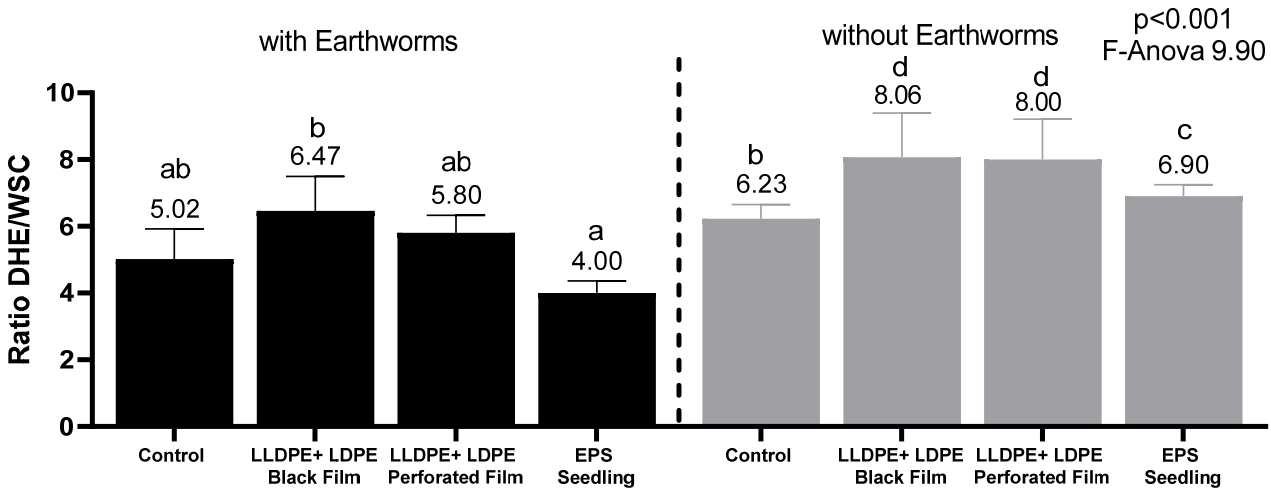

Figure 1. Graph of DHE/WSC ratio found in final vermicompost. Statical differences between treatments indicated by different letters at $p < 0.05$ (Tukeys and DMS test).

3.2. Vermicompost and Biofilm Exoenzymatic Activity

The enzymatic data obtained for the biofilm and vermicompost with different plastics are shown in Figure 2. The results for the vermicompost showed a significant difference in carboxylesterase (CbE) activity when compared to the control treatment without plastic. This behavior was observed in all the treatments, with and without earthworms (Figure 2a).

In all the cases under study, the treatments without EF presence (compost treatment) seemed to have more sensitivity to this CbE increase than vermicompost treatment with EF presence. In contrast, in the biofilm sample, the presence of EF appeared to promote an increase in the CbE enzyme compared to the biofilm sample without earthworms. Although a slight inhibition of the catalase enzyme was observed in the two kinds of plastic material tested (LDPE +LLDPE and EPS), the presence of plastic material did not significantly change the catalase activity compared to the control without plastic (Figure 2b). In the biofilm sample, the same behavior in all the treatments was observed. All the plastic treatments led to a sharp decrease in biofilm catalase activity compared to the substrate, with a mean decrease of 85% (Figure 2b). In our study, no significant differences among all the plastic treatments with earthworms and compost treatments without earthworms were shown by the ANOVA test for dehydrogenase activity (DHE) (Figure 2c). However, we highlight the increase in DHE activity observed in the compost treatment compared to the vermicompost, with 24.9, 37.3, and 53.3% increases for LDPE + LLDPE black film, LDPE + LLDPE perforated film, and EPS, respectively (Figure 2c). The biofilm did not show significant differences from the control treatments without earthworms.

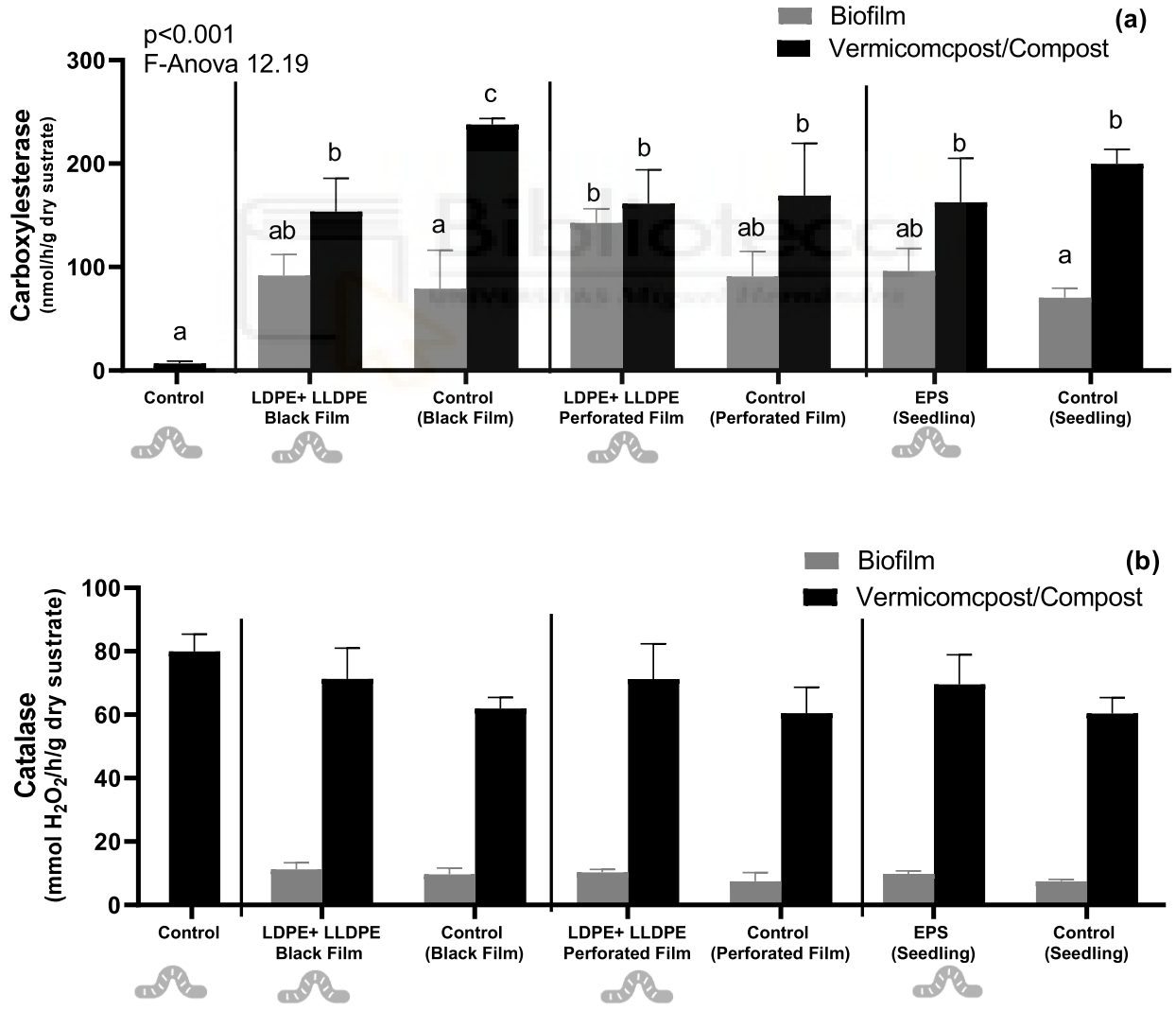

Figure 2. *Cont.*

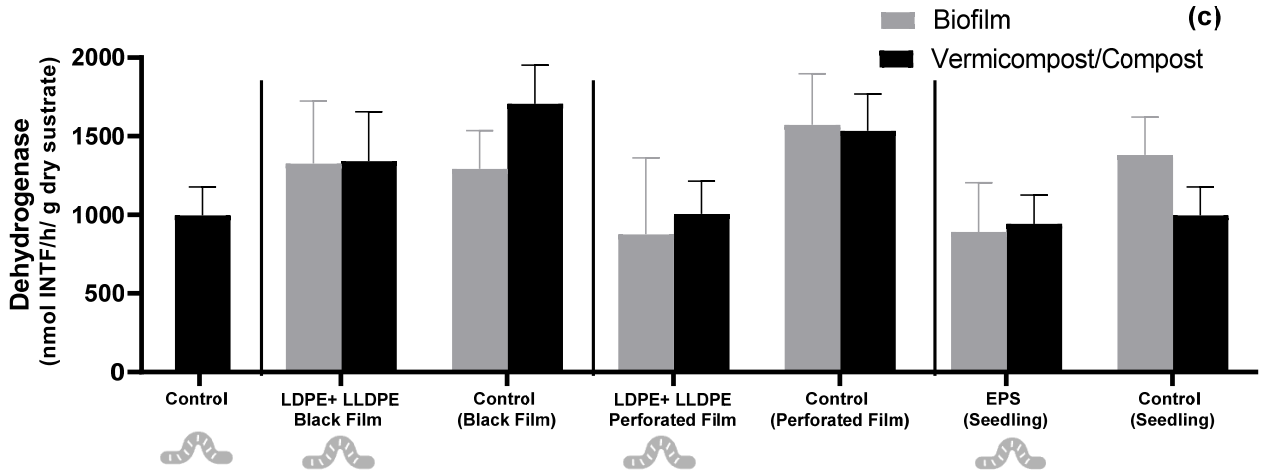

Figure 2. Graph of (**a**) carboxylesterase, (**b**) catalase, and (**c**) dehydrogenase activity determined in biofilm and vermicompost sample. The earthworm symbol indicates their presence in the vermicompost. Statical differences between treatments indicated by different letters at $p < 0.05$ (Tukeys and DMS test).

3.3. Eisenia fetida Survival and Body Weight

The control treatment maintained a higher density of earthworms with less mortality than the plastic treatments (Figure 3a). Therefore, the presence of APW seemed to decrease the survival rate of *E. fetida*. Significant effects were detected for the three different kinds of plastic tested. As shown in Figure 3a, earthworm mortality was observed mainly at the beginning of the microcosm bioassay. In the three plastic treatments, the rate of survival decreased in the initial stage, followed by stabilization until the end of the bioassay, except in the control treatment without plastic materials. At the end of the bioassay, the highest mortality was observed in the LLDPE + LDPE-black film treatment, with a decrease of 25% survival compared to the control (Figure 3a). Regarding the average body weight measured in all the specimens of each treatment, the control treatment enhanced *E. fetida* body weight compared to the plastic treatments, even though a higher density of earthworms was maintained with less mortality.

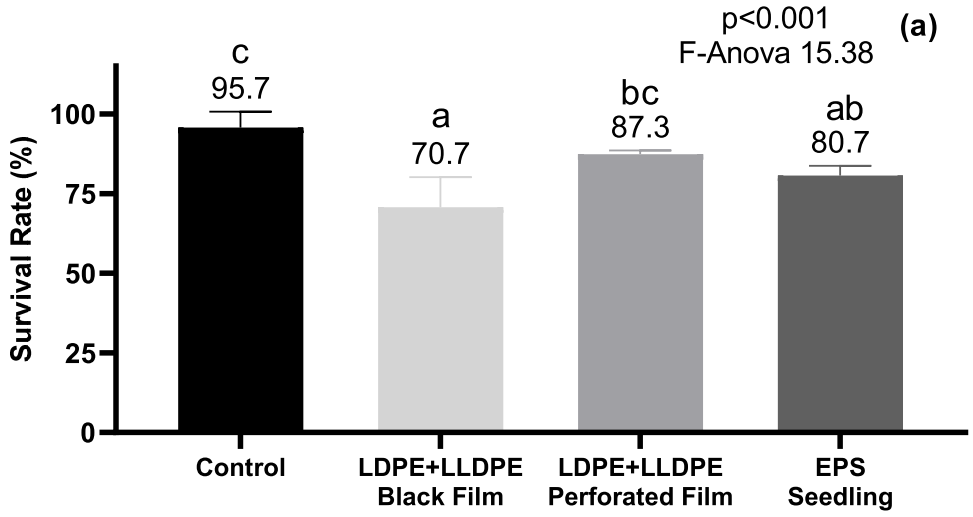

Figure 3. *Cont.*

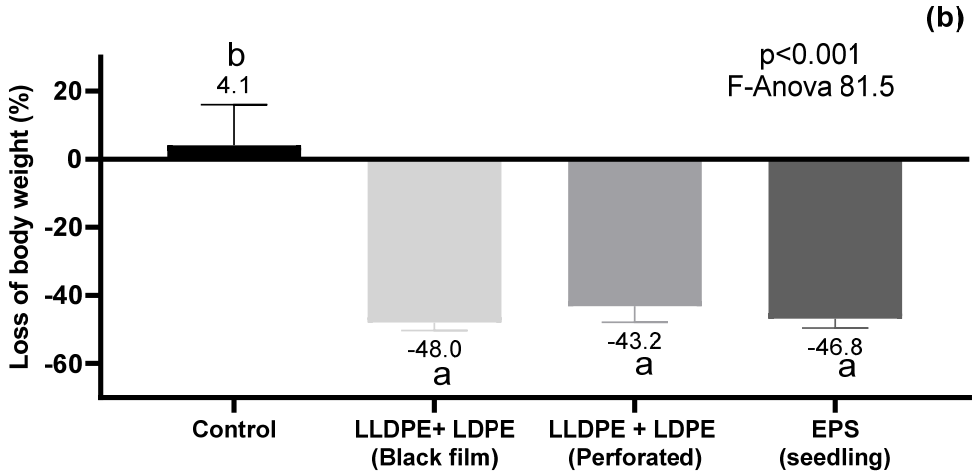

Figure 3. *Eisenia fetida* (**a**) survival and (**b**) loss of body weight. Statical differences between treatments indicated by different letters at $p < 0.05$ (Tukeys and DMS test).

3.4. Earthworm Biomarkers

Carboxylesterase is an esterase enzyme that plays a key role in the metabolic process of detoxification. It is considered an efficient protective mechanism for xenobiotic resistance in *Eisenia fetida* [35]. As shown in Figure 4a, a slight but insignificant increase in CbE activity was observed in all the treatments except EPS. Previous studies suggest that the luminal content of earthworms is the main source of CbE, which is released from the gut epithelium [36]. Due to the size and shape of the plastic tested in our study, the earthworms could not ingest the plastic. Regarding lipid peroxidation (Figure 4b), the results obtained are contrary to those expected, since we observed a decrease in lipid peroxidation. Our results indicate that the AChE in the *Eisenia fetida* exposed to LLDPE + LDPE and EPS were slightly affected compared to the control, although, as shown in the figure, not significantly (Figure 4c). The GST and GR activity in *Eisenia fetida* exposed to LLDPE + LDPE film plastic and EPS for 45 d was determined to characterize the effects of plastic on antioxidant defenses. In glutathione S-transferase, the response was a significant increase in earthworm body tissue activity after exposure to the plastic material (Figure 4d). The result of the glutathione reductase showed a significant change in earthworm exposure to LLDPE + LDPE in both the black film and perforated film, while EPS only caused a slight non-significant increase compared to the control treatment (Figure 4e).

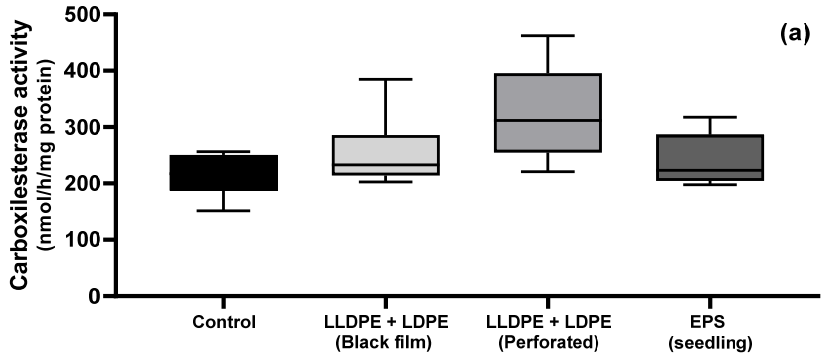

Figure 4. *Cont.*

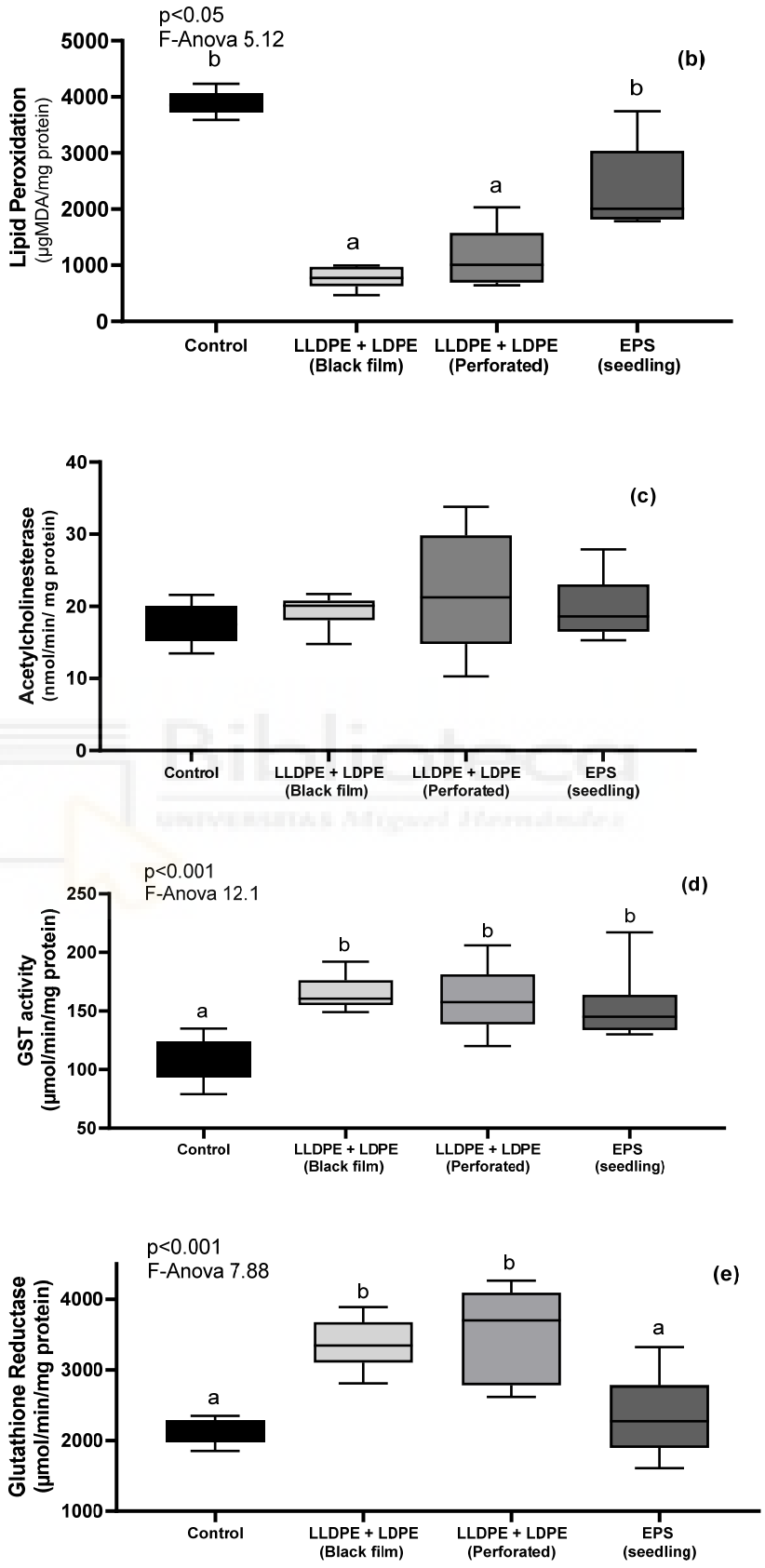

Figure 4. Carboxylesterase (**a**), lipid peroxidation (**b**), acetylcholinesterase (**c**), GST (**d**), glutathione reductase (**e**) activity in *E. fetida* body tissue. Statical differences between treatments indicated by different letters at $p < 0.05$ (Tukeys and DMS test).

4. Discussion

This study aims to gain knowledge about the effect of plastic presence in bio-waste during its treatment. The exposure bioassay of *Eisenia fetida* to APW presence was carried out with a concentration of plastic material (1.25% f.w.) that the European normative allows to be considered as compost fertilizer. The results obtained showed no significant effects on macronutrient NPK content. However, with plastic presence, significant changes were observed in other physicochemical characteristics of vermicompost such as WSC content or EC related to the degradation process. This affectation also was observed in vermicompost exposure to plastic waste, with an increase in CbE activity and remaining DHE activity. It is interesting to note the significant inhibition of CAT activity observed in biofilm samples, which we can hypothesize some organic plastic additives may have caused. In general, the result obtained seem to suggest a response of the microbiome to plastic exposure that leads to slowdown in the degradative process. Additionally, the earthworms showed negative morphological effects and mortality with the APW presence in feedstock. Additionally, the response of *E fetida* to plastic exposure show signs of oxidative stress, such as enhanced CbE activity or GST activity in body tissue induced by both types of plastic (EPS and LDPE + LLDPE).

An increase in pH values could be due to the microbiota, which utilizes the carbon fraction of the amino acids as an energy source and releases ammonia, causing an increase in pH (Table 2). In all the treatments with earthworms containing plastic, there were significant decreases in pH. Several reasons may explain this decrease: (1) the mucus from the *E. fetida* added to the ingested materials has been demonstrated to neutralize substrate [37]; (2) earthworms have shown excellent pH neutralization efficiency due to their calciferous glands [38]; and (3) the have an ability to regulate the release of organic acids depending on the characteristics of the starting feedstock [39]. pH values close to neutrality indicate the maturity of the vermicompost [40].

Increased EC in the vermicomposting process agrees with the findings by other authors [41,42] (Table 2). The reason for the rise in EC in the treatment with earthworms could be due to the higher mineralization of the organic matter, which released nutrient ions and soluble salts [43]. This is in contrast to the decrease in organic matter observed in the treatment without earthworms, but it could be explained by the ability of earthworms to promote some hydrolytic enzymes. These enzymes are not only linked to the C cycle (e.g., β-glucosidase), but also to N mineralization (e.g., urease) or the phosphorous cycle (phosphatase), which removes phosphate groups from organic matter [44]. The EC values in all the treatments exceeded the threshold of 4 dS m^{-1} (Table 2), which is considered a limiting factor for plant cultivation [45]. However, EC values below 8 dS m^{-1} are suitable for earthworm growth and development.

Earthworm mucus and activity are known to accelerate the rate of organic matter mineralization during the vermicomposting process, consequently leading to losses of total organic carbon by biotransformation [46,47]. The data obtained in this study did not show a great decline in organic carbon in the samples with earthworms. The final values of TOC content showed a significant difference between APW and earthworms, but no clear relationship between the presence of plastic and organic carbon evolution during the process was found. This drop in N in the sample with earthworms might be because parts of its initial content were transformed into body protein [48]. This behavior was also observed in the control treatment with and without earthworms, so it does not seem to be caused by exposure to plastic material. The mean values of total nitrogen (2.31%) were similar to those reported for other vermicompost (2.4%) made from agroindustrial waste, such as olive mill wastewater [31] (Table 2). The WSC content gradually decreased as vermicomposting progressed, in accordance with the consumption of available carbon sources for earthworm tissue formation and the subsequent stabilization of the substrate [30]. This behavior observed in LDPE + LLDPE film could be attributed to interactive mechanisms between this type of plastic material and the dissolved organic matter (DOM) content at a molecular level. Ref. [49] integrated spectroscopic methods into chemometric analyses, revealing the

microstructural exchange of DOM and plastic material, where polystyrene polymer-based plastic material interacted with the aromatic structure of DOM via a π–π conjugation. DOM was then trapped in the plastic polymers by carboxyl groups and C=O bonds, with the subsequent increase in concentrations corresponding to humic-like substances under pH conditions ranging from 7 to 9.

In general, the plastic samples without earthworms showed higher values of the DEH/WSC ratio (Figure 1) than the group with earthworms. This may indicate higher metabolic potential remaining in the feedstock without earthworms. The control treatments without plastic and with plastic, both in vermicompost with earthworms and compost without earthworms, had statistically higher values of DHE/WSC at the end of the bioassay (Figure 1). Therefore, the results suggest that the earthworms accelerated the hydrolytic phase of the feedstock, while the plastic caused a slowdown in the degradative gut processes of the microbiota and inherent microbiota in the compost/vermicompost.

The CbE response could be due to the gut-associated processes of the earthworms (Figure 2), which could have alleviated the increase of CbE on the substrate but caused higher concentrations of CbE activity close to the surface of the film as a detoxification response. Found in polluted soil, carboxylesterase enzymes are effective exoenzymes with the capacity to degrade a wide range of organic compounds [50]. This esterase is even an efficient mechanism for deactivating organophosphorus pesticides, because the pesticide remains irreversibly bound to the active site of the enzyme [51]. A previous study [49] about sewage sludge vermicomposting reported significant inhibition in catalase activity in treatments with high heavy metal content. In other studies on soil pollutants, ref. [52] found clear catalase activity inhibition in soil treated with pesticides (chlorpyrifos), suggesting that the response was associated with a change in microbial activity (Figure 2). Ref. [53] reported a negative effect on salt-affected soil in biochemical processes with a sharp decline in catalase. A possible reason for this reduction at lower levels could be that some plastic additives acted as catalase inhibitors. Hydroxylamine is widely used in mulch as a UV and light stabilizer, while resorcinol is an efficient gas barrier in several polymers [22]. In all the cases, the gut-associated processes of earthworms increased the release of catalase enzymes in the biofilm (mean value 10.4 mmol H_2O_2 h g^{-1} dry substrate), although not significantly compared to the control (mean value 8.1 mmol H_2O_2 h g^{-1} dry substrate) (Figure 2).

Increased DHE activity in the compost treatment compared to the vermicompost (Figure 2) might indicate remaining high metabolic activity in the control treatments without earthworms. This increase in DHE could indicate a lower stabilization in the compost samples caused by the higher degradative rates induced by earthworm gut-associated processes in the vermicompost. In addition, a slight difference was found between the control and plastic treatments with earthworms, with mean values of 995, 1281, 1148, and 875 nmol INTF h^{-1} g^{-1} for the control, LDPE + LLDPE black film, LDPE + LLDPE perforated film, and EPS, respectively. It is possible that the earthworms of the control treatment (without affectation for plastic presence) were able to consume the most available organic substances with the subsequent reduction in DHE enzyme activity. This seems to indicate that of the plastic material tested in this study, the LDPE + LLDPE, affected the earthworms' degradative capacity. The DHE behavior observed in the biofilm was similar to that observed in the vermicompost sample. This could be because of some available organic matter and microbial activity remained in the substrate attached to the plastics (Figure 2).

The effects observed in epigenic earthworms when they are exposed to plastic material under vermicomposting conditions have been described as a set of biotic factors involving various physiological processes, such as respiration rate, reproduction rate, feeding rate, and burrowing activity [33] (Figure 3). Their body weight showed that the nutrient capacity of the feedstock material was not a limiting factor, since earthworms consume half of their weight per day [54]. Therefore, we can assume that the weight loss in the plastic treatments was caused by stress in the earthworms' physiological activity. The maximum body biomass of *E. fetida* was reached at 21 days of bioassay in the control test vessel. In

contrast, earthworm body weight constantly decreased in all the plastic treatments during the study. No significant difference was detected between plastic materials. Similar values of negative weight variation were obtained for LLDPE + LDPE-black film, LLDPE + LDPE perforated film, and EPS seedlings at the end of the bioassay. No reproduction or cocoon presence was observed during the duration of the bioassay.

Increased CbE activity in *E. fetida* could be explained by the fact that worms must produce greater amounts of the protein α, β-hydrolase, which promotes CbE, to catalyze the hydrolysis of some xenobiotic compounds released from plastic debris (Figure 4). Recent studies have demonstrated that microplastics with varied chemical compositions can cause skin damage, tissue lacerations, immunity disruption, and neurotoxicity in terrestrial organisms such as ciliates, collembolans, and earthworms [55,56]. Some studies have even shown that *E. fetida* ingestion of MPs (HDPE, PP, and LDPE) smaller than 300 µm [57,58] leads to inflammatory processes between the gut epithelium and the chloragogeneous tissue, sometimes with the development of fibrosis and congestion [59]. Our results showed that the control treatment reached the highest value at the end of the exposure bioassay. All the plastic treatments, except EPS, followed the same trend, with low levels of lipid peroxidation. Previous studies have reported that MP size can significantly influence toxicity [60]. Lei et al. [61] found that the adverse effects of MPs were closely related to their size rather than their composition in zebrafish (*Danio rerio*) and in nematode such as *Caenorhabditis elegans*. Thus, we speculate that the mortality induced in *E. fetida* due to LDPE + LLDPE film plastic exposure led to low specimen density, which allowed the *E. fetida* to avoid this exposure and subsequent tissue damage. The size, high elasticity, and low rigidity of the polyethylene films tested support this hypothesis. Another possible reason could be the increased GST activity shown by *E. fetida* body tissue exposed to plastic material. Since this is an important antioxidant enzyme, it can scavenge lipid peroxides, thus contributing to reducing cellular oxidative damage [62]. In contrast to our results, the authors in [57] reported an increase in AChE in *E. fetida* exposed 21 and 28 days to 1.0–1.5 g kg^{-1} LDPE in soil, while the authors in [63,64] showed that AChE activity was inhibited in the dissected gut tissue of *Eriocheir sinesis* and *Pomatochistas microps* exposed to PS microplastics and PE, respectively. Refs. [65,66] also found a decrease in AChE. Ref. [65] indicated adverse effects in cholinergic neurotransmission and, thus, possibly in the nervous and neuromuscular functions of juvenile fish (common goby—*Pomatoschitas microps*) following exposure to polyethylene microplastic (1–5 µm). They observed a 42% inhibition in brain AChE. Ref. [66] reported AChE inhibition in *Eisenia andrei* exposed to polystyrene–HBCD in soil after 7 d of exposure, showing a recovery to normal values after 28 d of exposure. As studies that investigated the same type of plastic obtained different results, this might also indicate varying types of action depending on several factors, such as the type of plastic, but also concentration, shape, size, and the potential influence of additives. Therefore, the action mechanism of plastic material on AChE is still not clear, but we can assume that the concentration of plastic (1.25%), as well as the size or shape of the plastics tested seemed to affect the low acute toxicity of *E. fetida*. The same behavior shown in regarding GST activity was observed by [66] in *Eisenia andrei* exposed to polystyrene–HBCD and car tire abrasion plastic present in soil. Furthermore, they reported a time-dependent response with increased GST activity from day 7 to day 28 of exposure. On the other hand, ref. [67] reported significantly inhibited GST activity in *E. fetida* after exposure to HDPE and PP microplastics for 14 days. A similar decreasing trend for GST activity was observed in *E. fetida* when exposed to low-density polyethylene (LDPE) and PS MPs [68]. Other studies have also reported that exposure to plastic material can upregulate the level of reactive oxygen species (ROS), thereby perturbing the antioxidant system [69,70]. Therefore, the immune response against ROS is a mechanism that requires an action–response balance. Exposure to LDPE + LLDPE or EPS resulted in the accumulation of ROS, which then stimulated the biosynthesis of antioxidant enzymes. Once the excess of accumulated ROS overwhelms the antioxidant defense systems, the synthesis or structure of antioxidant enzymes can be easily influenced, resulting in a decrease in enzyme activity [71]. Another

reason for the differences found in GST activity is the production of malondialdehyde, also known as a thiobarbituric acid reactive agent, as a product of lipid peroxides. This carbonyl compound is one of the most abundant end-products of lipid peroxidation and may have induced the GST activity through its elimination by conjugation with GSH [72]. The glutathione-dependent enzyme is related to a mechanism of ROS–GSH balance. These increases in GR activity could be caused by an antioxidant response.

5. Conclusions

The findings of this study suggest that the presence of LDPE + LLDPE and EPS (1.25% f.w.) in bio-waste allows their bio-treatment throughout composting or vermicomposting. However, signs of degradative process slowdown were observed in the enzymes measured, which can lead to a retardation of the hydrolytic phase. Nevertheless, the final characteristics of the vermicompost exposed to plastic did not show significant differences from the control vermicompost. Two types of plastic tested had a negative morphological effect and even mortality on *E. fetida*. The measured biomarkers reflected an antioxidant response through enhanced GST activity and a detoxification process through increased CbE in earthworm tissues. These results have extended our knowledge about the effects of agricultural plastic waste on bio-waste treatment. However, further future studies should include a wide variety of plastic types, concentrations, sizes, and shapes to better understand the mechanisms involved in oxidative stress.

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Effect of agricultural microplastic and mesoplastic in the vermicomposting process: Response of *Eisenia fetida* and quality of the vermicomposts obtained☆

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ABSTRACT

This work evaluates the effect of agricultural plastic waste (APW) in two particle sizes, microplastic and film debris, and subjected to a pre-treatment by exposure to UV-C, in the development of the vermicomposting process. *Eisenia fetida* health status and metabolic response and the vermicompost quality and enzymatic activity were determined. The environmental significant of this study is mainly related to how can affect plastic presence (depending on plastic type, size and/or if it is partially degraded) not only to this biological process of organic waste degradation, but also to the vermicompost characteristics, since these organic materials will be reintroduced in the environment as organic amendments and/or fertilizers in agriculture. The plastic presence induced a significant negative effect in survival and body weight of *E. fetida* with an average decrease of 10% and 15%, respectively, and differences on the characteristics of the vermicomposts obtained, mainly related with NPK content. Although the plastic proportion tested (1.25% f. w.) did not induce acute toxicity in worms, effects of oxidative stress were found. Thus, the exposure of *E. fetida* to AWP with smaller size or pre-treated with UV seemed to induce a biochemical response, but the mechanism of oxidative stress response did not seem to be dependent on the size or shape of plastic fragments or pre-treated plastic.

Environmental implication

The main objective of this study was to evaluate the effect of agricultural plastic waste (microplastic and film debris) during the vermicomposting process. In addition, the plastic material was pre-treated by exposure to UV-C to simulate the natural weathering and partial degradation of plastic polymers in the environment. The environmental implication of this work is mainly related to how can affect the plastic presence, depending on the plastic type, the size and/or if it is partially degraded, to the biological process of degradation of organic wastes as vermicomposting and to the quality of the vermicomposts obtained, since these organic materials will be reintroduced in the environment as organic amendments and/or fertilizers in agriculture. Several previous works have reported the effect of microplastic during vermicomposting, but there is little information concerning how can affect to the process specific characteristics of the material on the whole, such as type of plastic, size and previous degradation on the vermicompost characteristics and on *Eisenia fetida* response and health status using indicators of the oxidative stress apart from survival and body weight.

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1. Introduction

Currently, the use of plastics is extended in European agriculture, due to their contribution to the increase in the quality and the quantity of crop production, but their end-of-life management constitutes an environmental problem due to the usual incomplete recovery after the crop season. To produce flexible, semi-rigid and/or rigid materials, the main polymers used in agriculture are the polyethylene (PE) and the polyethylene terephthalate (PET), due to their high impact resistance, low cost, good workability, and optimal chemical resistance properties. Low-density polyethylene (LDPE) and linear low-density polyethylene (LLPDE) are principally used to obtain films (for greenhouses, low tunnels, mulching, and silage), due to their elasticity and high tear and impact strength (Dorigato et al., 2011). The most abundant and problematic types used in agri-food industries that comprise >60% of the agro-industrial sector, are still largely considered to be non-biodegradable (Inderthal et al., 2021). In 2019, the global production of plastic material was around 370 million tons, of which only 9% was recycled, 12% was incinerated and remaining left in the environmental or landfill (Kumar et al., 2021). Thus, one of the production sectors with higher mulching plastic consume is the agricultural sector. In this sense, Zhang et al. (2020) estimated that largest use of plastic mulch in agricultural system led to an accumulation of 550,800 tons of plastic residues in soil per year.

Plastic degradation of plastics comprises any change of the physical or chemical properties. It can occur because of biological, chemical or physicochemical processes and when this alteration is irreversible over time is known as ageing. In the environment, these changes can lead to microplastic formation by fragmentation of larger plastic (mesoplastic) by exposure to weather conditions (Jiang, 2018; Sobhani et al., 2022). Thus, due to their progressive fragmentation into smaller size particles, the plastic wastes from the agro-industrial sector have demonstrated to be a source of plastic debris in multiple environmental compartments, to be atmospherically long-transported, or even incorporated in the trophic chain (Allen et al., 2019). This increasing in dispersion of plastics in multiple ecosystems has led to their accumulation in bio-wastes of several streams, such as municipal solid waste (Li et al., 2022), sewage sludge (Gao et al., 2020), animal manure (Wu et al., 2021) or agricultural organic wastes and their derived compost (Weithmann et al., 2018). Currently, there is a lack of studies about the quantity and different type of plastic presence in organic wastes on a global scale. Thus, several local studies have demonstrated the accumulation of plastic debris of multiple size and shape in biowaste (Gui et al., 2021; Pathan et al., 2020).

Verrmicomposting, as a single treatment or combined with composting as pre-treatment, is an alternative widely used for the valorisation of a wide variety of organic wastes, which allows to obtain a stabilized organic with a fertilizer value, which can be used in agriculture as organic amendment. Several studies suggest that earthworms interact with microplastics in soil (Huerta Lwanga et al., 2016, 2018; Rillig et al., 2017; Hodson et al., 2017). However, these studies have been performed using soil-dwelling earthworms in terrestrial ecosystems (Rillig et al., 2017; Rodríguez-Seijo. et al., 2018; Li et al., 2018), and comparable interaction should be investigated in vermicomposting, with epigeic earthworm species such as *Eisenia fetida*. Moreover, the studies carried out to evaluate the effect of the presence of plastic material using these annelids have been focused on microplastic (MP) and nanoplastic (NP) size particles (Rodríguez-Seijo et al., 2018; Jiang et al., 2020; Lackmann et al., 2022). Although some studies have shown that during the fragmentation process various types of associated plastic chemical can be detected (Sobhani et al., 2022), often no attempt is made to evaluate the potential toxicity of large plastic such as film debris commonly defined as mesoplastic (fragments of 1 to <10 mm size). In addition, Pathan et al. (2020) found that non-compostable plastic material can be as a microhabitat, which is quickly colonized by microorganisms, forming the plastisphere, a dense biofilm on the surface of the plastic. Furthermore, Jing et al. (2014) reported a mechanism of passive protection of biofilm against plastic particles in soils, which are dependent on the biofilm matrix characteristics and capable of entrapping and binding chemical by-products into the biofilm surface layer.

Thus, this work aims to evaluate the influence of different agricultural plastic wastes, at different particle sizes (microplastics and mesoplastics) and subjected to a pre-treatment by exposure to UV-C (250h) to simulate environmental ageing, during the vermicomposting process to evaluate the potential adverse effects on the quality and enzymatic activity of the vermicompost obtained and on *Eisenia fetida* response and health status.

2. Material and methods

2.1. Feedstock and AWP characteristics and biofilm samples

The feedstock used in the experiment was an organic material from agro-industrial wastes pre-treated by a partial composting to degrade toxic compounds for earthworms, such as ammonium and considered in a previous experiment. This stabilized organic material was obtained after an aerobic turning process that lasted 96 days, from a mixture of four ingredients (khaki pruning waste, agri-food sludge from citric industries, goat manure and vineyard pruning) in a proportion of volume of 45:35:15:5, respectively. A more detailed description of the process can be found elsewhere (Sáez et al., 2022). This material was selected for the experiment due to the absence of plastic materials in the raw materials used and during the stabilization process.

Five different APW materials frequently used in different agricultural practices were considered: low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), LLDPE + LDPE, polyethylene terephthalate (PET) and polystyrene (PS). LLDPE and LLDPE + LDPE were provided by two Spanish private companies (Repsol S.A. and Solplast S.A.), while the rest of plastics were provided by a public entity (University of Pisa, UNIPI). Additional information regarding the characteristics of the APW selected are shown in Table S1. The variables considered, apart from the type of plastic type, were: a) particle size of the APW material (two different particle sizes were assessed and selected as representative of the size of mulching film pieces found in the environment after natural ageing, such as microplastic-MP (1–1000 μm) and film debris-mesoplastic (fragments 1 to <10 mm) with pieces of approx. 1 cm^2); b) a pre-treatment based on the exposure of the plastics to ultraviolet light type C for 250h in order to simulate the natural exposure to the weathering conditions (natural ageing). For the UV pre-treatment, a close chamber (0.25 m^2) with reflective walls and 2 UV-C lamps at 253 nm (Philips TUV T8 F17 1SL/25) were used, where the APW material was exposed to irradiation for 250 h at 137 W/m^2. Also, continuous ventilation was installed in order to maintain the temperature in a range from 15 to 25 °C.

The mesoplastic pieces were obtained using scissors to cut the AWP material into small pieces with irregular shape, whereas for the microplastic (MP) size, the plastic in pellet format were mechanically grinded in a rotor paddle Mill (RETSCH Mill SK100 Comfort) and then sieved. In order to discriminate the affectation of biofilm formation in the microbial community, further enzymatic activities have been also measured. For the obtaining of the biofilm samples, the small APW pieces were carefully separated from the substrate and scraped with a spatula. Later, the sample was homogenized and 0.1 g were suspended with 15 ml of distilled water ratio 1: 15 (w/v), being kept in refrigerator at 4 °C until determination of the enzymatic activities.

2.2. Experimental design

In order to reproduce the conditions of the vermicomposting process of agro-industrial organic wastes with plastic presence at laboratory scale, five different APW materials, pre-treated or not with UV light and at different sizes (mesoplastic and microplastic) were used. The

Table 1
Effect of the type of AWP material and *Eisenia fetida* presence in the physico-chemical and chemical parameters of the organic material obtained at the end of the bioassay.

		pH	EC (dS m^{-1})	OM (%)	TOC (%)	TN (%)	P (%)	K (%)	WSC (g kg^{-1})	C_{FA} (%)	C_{HA} (%)
Main effects											
***E. fetida* presence**	Yes	7.47 a	4.66 b	53.8 a	26.5 a	2.36 a	0.74 a	1.16 a	8.19 a	2.52 a	3.23 a
	No	7.86 b	3.88 a	54.6 b	26.9 a	2.34 a	0.75 a	1.17 a	9.57 b	2.54 a	3.49 b
Type of AWP	LDPE	7.68 b	4.44 b	53.0 ab	28.4 b	2.35 a	0.72 b	1.19 b	7.66 ab	2.34 bc	3.06 b
	LLDPE	7.67 b	4.22 ab	53.8 ab	27.4 ab	2.33 a	0.76 bc	1.22 b	9.29 bc	2.75 b	3.96 b
	PET	7.49 a	4.23 ab	54.3 b	25.0 ab	2.40 a	0.61 a	0.78 a	7.61 ab	1.62 a	1.56 a
	PS	7.48 a	4.23 ab	54.0 ab	25.0 ab	2.35 a	0.63 a	0.79 a	7.18 a	1.58 a	1.88 a
	LDPE + LLDPE	7.73 bc	3.84 a	53.9 ab	24.6 a	2.31 a	0.81 c	1.21 b	9.09 bc	2.91 c	3.75 b
***E. fetida* presence**	**Type of APW**										
Yes	Control without AWP	7.48 ab	5.07 d	52.0 a	25.3 a	2.41 ab	0.76 a	1.24 a	8.22 a	2.93 a	3.75 a
	LDPE	7.50 ab	4.84 cd	52.1 a	27.8 a	2.44 ab	0.72 a	1.19 a	7.22 a	2.27 a	3.04 a
	LLDPE	7.52 b	4.42 bc	53.8 ab	27.3 a	2.30 a	0.74 a	1.22 a	8.83 a	2.64 a	3.70 a
	PET	7.27 a	4.66 c	53.2 ab	24.7 a	2.45 ab	0.64 a	0.81 a	7.36 a	1.64 a	1.28 a
	PS	7.42 ab	4.37 bc	52.9 ab	24.8 a	2.39 ab	0.60 a	0.73 a	6.82 a	1.71 a	1.64 a
	LDPE + LLDPE	7.37 ab	4.45 bc	55.0 b	25.2 a	2.29 a	0.72 a	1.24 a	8.35 a	2.65 a	3.47 a
No	Control without AWP	7.88 bc	4.17 bc	55.3 b	27.3 a	2.53 b	0.72 a	1.40 a	8.85 a	2.57 a	3.16 a
	LDPE	7.85 c	4.04 ab	54.0 ab	29.1 a	2.27 a	0.72 a	1.19 a	8.11 a	2.42 a	3.08 a
	LLDPE	7.82 bc	4.01 ab	53.9 ab	27.5 a	2.36 ab	0.78 a	1.28 a	9.73 a	2.86 a	4.21 a
	PET	7.71 bc	3.90 ab	55.4 b	25.3 a	2.31 a	0.59 a	0.75 a	7.86 a	1.61 a	1.85 a
	PS	7.53 b	4.08 b	55.1 b	25.3 a	2.31 a	0.65 a	0.85 a	7.54 a	1.43 a	2.11 a
	LDPE + LLDPE	8.10 c	3.22 a	52.8 ab	24.1 a	2.32 a	0.81 a	1.18 a	9.82 a	3.17 a	4.11 a
Statistical significance											
	E. fetida presence	***	***	*	ns	ns	ns	ns	*	ns	*
	Type of APW	***	***	***	***	ns	**	**	***	***	***
	E. fetida x APW	***	***	***	ns	**	ns	ns	ns	ns	ns

EC: Electrical conductivity, TOC: Total organic carbon, OM: Total organic matter, WSC: water-soluble carbon, C_{FA}: fulvic acid-like C; C_{HA}: humic acid-like C. n.s.: not significant $P > 0.05$; *, **, ***: significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Average values in a column followed by the same letter are not significantly different at $P < 0.05$ (Tukey-b post-hoc test).

earthworms used belong to the specie *Eisenia fetida,* selected for this study due to this epigeic specie is commonly used for vermicomposting approaches (OECD, 2016). The procedure of obtaining and preparation of the earthworms for the bioassay are detailed in a previous work (Sáez et al., 2022). Considering the main factors established in the experiment (plastic type, pre-treatment or not and particle size, *E. fetida* presence and AWP presence), three different types of experimental devices with three replicates each were prepared: a) feedstock + AWP without earthworms; 2) feedstock + earthworms without AWP presence; c) feedstock + APW + earthworms.

The bioassay consisted in an incubation during 45 days in Petri dishes (15 cm ø) (Domínguez, 2018) using 80g of feedstock adjusted with distilled water to 70% of moisture content. Then, 1g of APW material was added per replicate (1.25% f. w. proportion) (Sáez et al., 2022). The incubation containers were kept into isolated chambers under controlled conditions (20° ± 2 °C and darkness). After 7, 21, 30 and 45 days of exposure, survival and body weight variation were determined. For this, earthworms were carefully extracted from the Petri dish of each replicate by hand sorting, counted for survival, weighted and recorded to obtain the mean body weight of each treatment. During the exposure time of bioassay, when mortality was observed, the worms were immediately removed from the Petri dish. At the end of bioassay, the final materials obtained, after the bioassay without earthworms (hereinafter referred to substrate) and with earthworms (vermicompost) from each replicate were homogenized and the samples were divided in two subsamples: one was used immediately for moisture determination and freeze at −80 °C until enzyme activity determination and the other were partially air dried in oven equipped with forced aeration at 60 °C. To obtain a dust particle size the sample was ground using an agate ball mill (RESTCH mod. MM400) and dried at 105 °C for further determinations. In addition, microscope images were obtained using a Camera MOTICAM S3 connected to Trinocular Microscope MOTIC® SMZ 140/143to compare the effect of the bioassay in the plastic size and shape (images display in S3 as Supplementary Material).

2.3. Analytical methods

2.3.1. Physico-chemical and chemical characteristics of the substrate and vermicompost

The physico-chemical and chemical parameters in the substrate (after the bioassay without earthworms) and vermicompost samples were determined according to the methods described by Sáez et al. (2022). Briefly, water-soluble extracts (1/10, w/v) were analysed for the physico-chemical parameters (pH and electrical conductivity); total organic carbon (TOC) and total nitrogen (TN) were determined using an automatic elemental micro-analyser; total organic matter (OM) was evaluated by loss on ignition (430 °C for 24 h). Moisture content was determined by drying at 105 °C for 24 h, whereas macronutrients and micronutrients (P, K, Ca, Cu, Mg, Fe, Mn and Zn) and toxic heavy metals (Cr, Ni, Cd, Hg and Pb) were determined in the extract obtained after the acid digestion (HNO_3/H_2O) (1:1 v/v) using a microwave by ICP-OES. The 0.1 M NaOH-extractable organic carbon (Cex), fulvic acid-like carbon (C_{FA}), humic acid-like carbon (Cha) and water-soluble C were determined with an automatic carbon analyser for liquid samples (TOC-V CSN Analyser).

2.3.2. Enzymatic activity of substrate, vermicompost and biofilm and earthworm biomarkers

The enzymatic activities (carboxylesterase (CbE), catalase and dehydrogenase (DHE)) were determined using the microplate-scale format protocols described in detail by Sáez et al. (2022) in an aqueous suspension 1:50 (w/v) of the substrate and vermicompost samples, respectively, while for the biofilm, the sample separated from the substrate or vermicompost was previously homogenized in the water-soluble ratio 1:10 (w/v).

For the determination of the biomarkers (total protein content, CbE determination and lipid peroxidation), six earthworms randomly selected from each replicate were selected and 24 h without feedstock in order to eliminate their gut content. Later, the body tissue of the earthworms was homogenized to obtain the post-mitochondrial fraction which was aliquoted and stored at −80 °C until analysis. This procedure

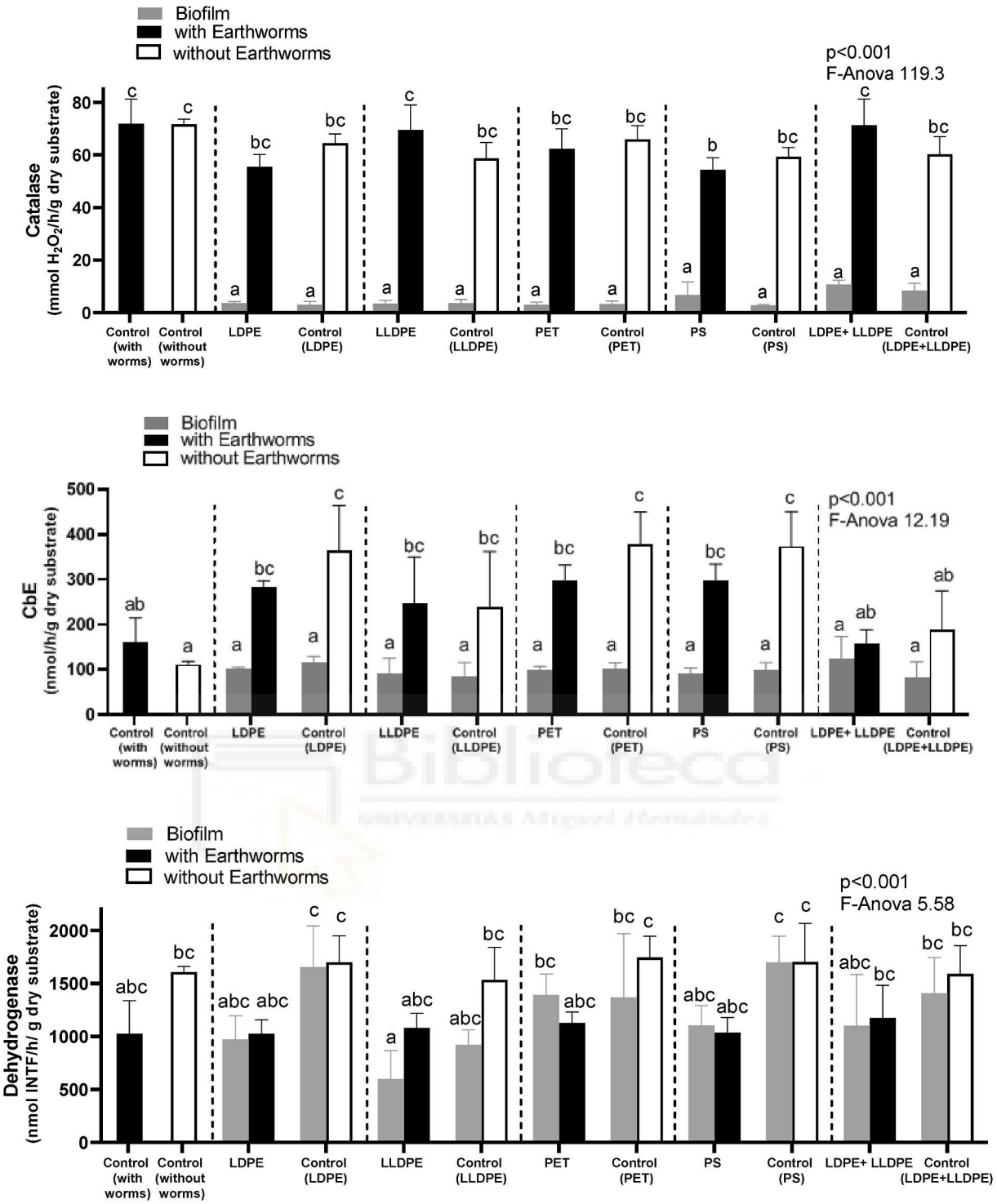

Fig. 1. Enzyme activities (catalase, carboxylesterase (Cbe) and dehydrogenase) measured in biofilm, substrate without earthworms and vermicompost. Different letters indicate significant statistical differences ($P < 0.05$).

and those for the determinations of the biomarkers can be found elsewhere (Sáez et al., 2022).

2.4. Statistical analysis

The statistical analyses were based on an ANOVA analysis to study the effects of the treatments in the environments with and without earthworms and the multivariate general linear model (GLM), to assess the effects of the five variables considered (*E. fetida* presence, APW type, APW presence, APW size and pre-treatment). In both statistical analyses, the Tukey-b was used as post-hoc test. The statistical analyses were conducted using the IBM SPSS Statics V.28 software package.

3. Result and discussion

3.1. Effect of the AWP type and/or E. fetida presence on the chemical characteristics of the end-products obtained

After 45 days of bioassay, the pH values were significantly lower in the vermicompost and with APW compared to those with APW and without earthworms (Table 1). This could be due to the release of volatile fatty acids as a consequence of the higher organic matter degradation in these treatments. On the other hand, *E. fetida* mucus is added to the ingested materials, leading to neutralize the substrate (Pérez-Godínez et al., 2017). Moreover, earthworms have shown an excellent pH neutralization efficiency due to their calciferous glands (Mubeen and Hatti, 2018) and the ability to regulate the release of organic acids depending on the characteristics of the starting feedstock (Angst et al., 2019). The type of AWP also showed a significant effect on the pH, showing the plastics PET and PS the lowest pH values. However, in the substrate without earthworms, compared with the initial substrate, no differences were found induced by the plastic presence.

E. fetida presence induced higher electrical conductivity mean values, also observing higher EC values in the treatments with AWP and earthworms (Table 1). The EC values obtained in all treatments with earthworms exceeded the threshold of 4 dS m^{-1}, limiting value for plant cultivation in soilless crops (Lasaridi et al., 2006), but within the recommended ranges for their use as organic amendments. On the other hand, EC values below 8 dS m^{-1} are adequate for earthworm growth and development (Rahimi and Karimi, 2016). This EC increase in vermicomposting processes has been reported in previous works (Khalil and Sanaa, 2009; Fernández-Gómez et al., 2010). The higher EC values observed in the treatment with worms can be attributed to the increased organic matter mineralization, which releases ions (cations and anions), unavailable nutrients in more available forms and the production of salts, ammonium and inorganic (soluble salts) (Bernal et al., 2009). This behaviour coincides with the greater decrease in the organic matter concentrations observed in the treatment with worms, which might also be explained by the ability of the worms to promote some hydrolytic enzymes, not only linked to the C cycle (β-glucosidase or carboxylesterase), but also related with other macronutrients, such as the phosphorus cycle (phosphatase) that remove phosphate groups from organic matter (Nogales et al., 2008) or related with N mineralization (urease, protease). The type of AWP also influenced this parameter, showing the treatments with the mixture of LDPE + LLDPE the lowest salinity mean values.

The strong reduction in the OM and TOC contents observed at the end of bioassay can imply an accelerated mineralization of nutrients bound to organic matter. However, the presence of earthworms and the combined factors (*E. fetida* and AWP presence) did not affect the evolution in TOC content, the opposite effect being observed for OM. In addition, with the presence of APW was observed a lower TOC reduction, especially in LDPE + LLDPE, this could be indicative of a slight antimicrobial effect on substrate. The type of AWP had a significant effect, showing PET the highest OM mean contents at the end of the bioassay, probably due to the different structure of this polymer, which could favour the retaining of substances with an organic matrix (Fadare et al., 2019).

In general, the total nitrogen (TN) tended to increase in all treatments compared with the initial feedstock, with and without earthworms, only founding a significant effect of the combined factors (*E. fetida* and AWP presence). In other studies of vermicomposting, an increase in TN has been reported due to bioconversion process of decomposition of waste by earthworms (Cynthia and Rajeskhumar, 2012). Also, trough microbial mediated nitrogen transformation results in further increase in nitrogen (Suthar and Singh, 2008).

Regarding to P and K contents, no statistical differences were found when the treatments with and without earthworms were compared, as in the case of the combined effect of *E. fetida* and AWP. However, the differences in the P and K contents seem to be related to the type of APW. Total K and P levels were significantly lower in PET and PS. As it has been previously observed in the OM, the type of structure of these polymers could have influence OM degradation, since it has been reported the capacity of polystyrene-based plastics to remain bound and retain substances with an organic matrix (Fadare et al., 2019), which avoids their decomposition and released of nutrient bond. Thus, the results obtained seem to indicate that the differences observed in NPK contents could be mainly related with the type and composition of APW.

In the WSC contents, a significant effect of *E. fetida* and of AWP presence was observed, but this effect was not significant when both factors were combined. The presence of the earthworms significantly reduced the WSC contents, this result being previously reported by other authors, since the gut associated processes and microorganisms consume labile forms of organic matter as carbon source in their tissue formation, resulting in a reduction in WSC content during vermicomposting (Yadav and Garg, 2011). Concerning the effect of the type of AWP, in general, the treatments with LLDPE polymer in their composition presented higher values of WSC. The same behaviour was observed in the treatments with and without earthworm presence for LLDPE. Thus, one potential reason for this is that the polymer backbone of LLDPE partially retains and bounds with organic matrix, avoiding its consumption, as it was found by Chen et al. (2018) in a study about the interaction of MP with the aromatic structure of DOM. The type of AWP had a significant effect on the humic and fulvic acid-like C contents, but the *E. fetida* presence only affected significantly C_{HA}. In both parameters (C_{FA} and C_{HA}), the combination of *E. fetida* and AWP presence did not show a significant effect, while the treatments with PET and PS showed again the lowest values. This reduction in humic compounds contrasts with that would be expected in vermicomposting process. However, other studies about plastic presence in soil have reported a decrease in humic acid-like compounds due to strong adsorption capability to polystyrene nanoplastic particles (Velzeboer et al., 2014; Cai et al., 2018).

3.2. Effect of APW presence on the substrate, vermicompost and biofilm enzyme activity

Catalase enzyme plays a key role in antioxidant system to response to oxidative stress, preventing oxidative damage (Giulia et al., 2012). In general, a slight inhibition of the catalase (CAT) activity was found in all the treatments with APW presence compared with the control treatment in both cases, with and without earthworms (Fig. 1). In presence of the earthworms, only the treatments with PS showed statically lower values than the control treatment, showing the rest of AWP treatments LDPE and PET values statistically similar or slightly lower than the control treatment. A potential reason for the observed fall at these low levels could be due to the ability of some plastic additives to act as CAT inhibitors. Additives such as hydroxylamine and metallocenes are widely used as UV and light stabilizers in the film manufacturing process, while resorcinol is an efficient gas barrier of several polymers (Rodríguez-Seijo et al., 2018). These compounds show affinity for binding catalase enzyme site and leads the enzyme to permanent inactivation (Pritchard, 1998; Rodríguez-Seijo et al., 2018). Thus, it seems to the presence of APW in the proportion tested (1.25% f. w.) lead to a partial inhibition in CAT production, but not induce a clear detoxifying response in *E. fetida*. In the biofilm samples, all APW treatments led to a sharp reduction in the CAT activity in relation to the substrate, with a mean decrease of 85% (Fig. 1).

Concerning to the carboxylesterase (CbE) activity, in general, the different AWP treatments seemed to induce an increase in the values of the CbE activity, especially in absence of *E. fetida,* with the values closer to the control treatment with the LDPE + LLDPE presence. The presence of LDPE, LLDPE, PS and PET during assay led to a significate increase in CbE activity compared with the control treatment without plastic with a mean increase of 39.7%. When the control treatment without AWP is compared with the presence and absence of earthworms, an increase in

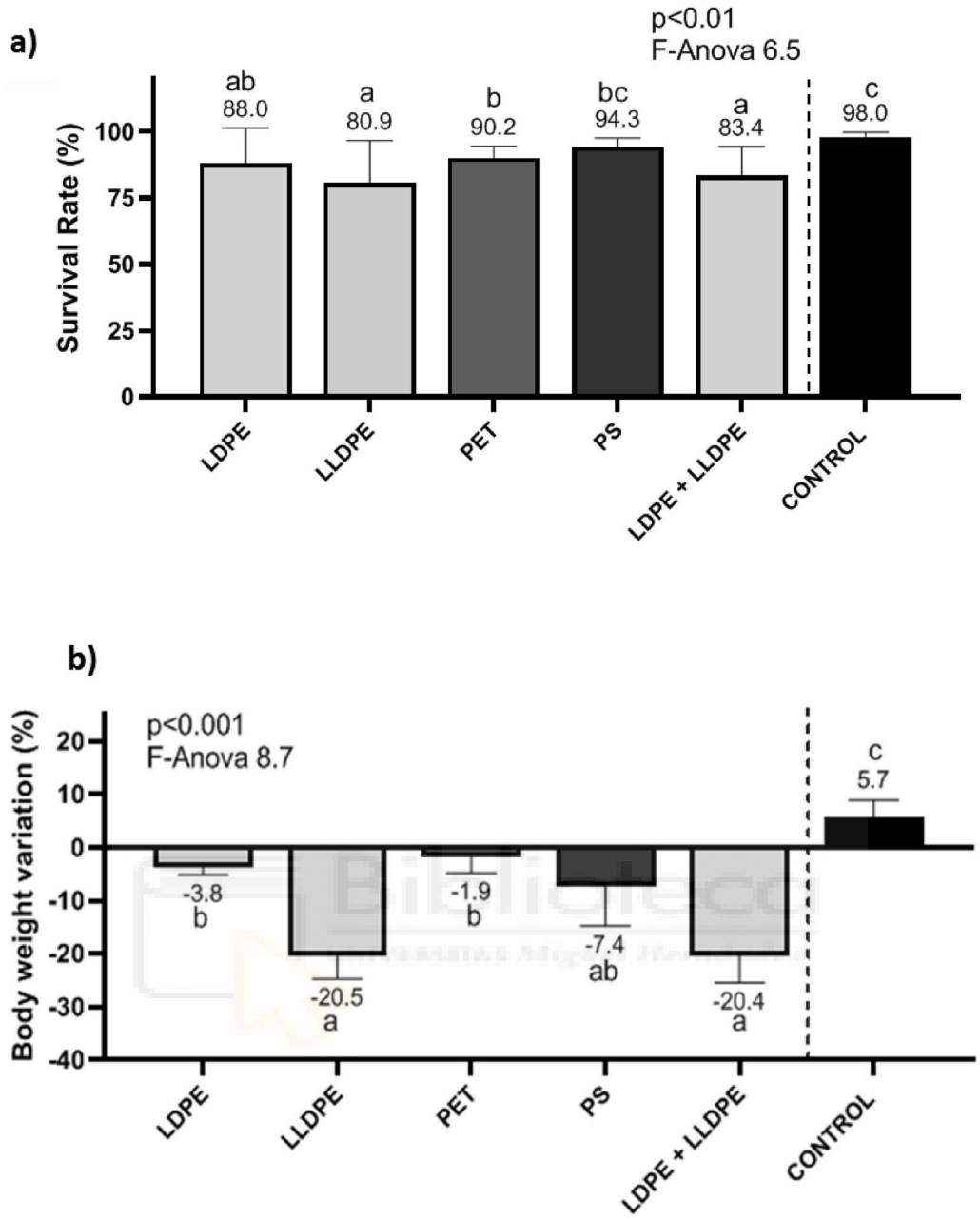

Fig. 2. a) Survival and b) body weight variation in *Eisenia fetida*. Different letters indicate significant statistical differences ($P < 0.05$).

CbE activity is observed, probably due to this esterase is mainly secreted in the intestinal lumen of the earthworms themselves (Sánchez-Hernandez et al., 2009) and therefore, earthworms casting may act as source of stable and active CbE activity (Sánchez-Hernández et al., 2015). This increase in presence of plastic materials could be due to the occurrence of cellular damages, which may produce changes in energy consumption to counteract the effects imposed by oxidative stress or other mechanisms (Rodríguez-Seijo et al., 2018). In the biofilm samples, all samples obtained final values close to the control treatment without earthworms, not being affected for earthworms and/or APW presence, independently of the type of plastic material. The strong inhibition observed could indicate a bioscavenging role of this esterase in response to close contact with APW material. In soils have been described a mechanism of passive protection of biofilm against plastic particles, which are dependent on the physical-chemical properties of the biofilm matrix (Jing et al., 2014). Unfortunately, no data are available on the effects of MPs or film debris (mesoplastics) in organic waste biofilm formation.

The DHE activity is directly related with biological oxidation of organic matter. The type of AWP seemed not to induce a significant effect on the DHE activity in the environment with *E. fetida*, observing the same values as for the control treatment (Fig. 1). However, without earthworms, a slight increase was observed for all the AWP treatments, especially for LDPE, PET and PS. These results seem to indicate that the digestive system of earthworms was able to break down the organic matter in the feedstock with APW, increasing the particle surface-to-volume ratio, and thereby the maintenance the number and activity of the microorganisms. Moreover, the higher DHE activity found may be due to a lower stabilization reached in these samples. This is corroborated by the higher WSC content remained in samples without earthworm presence. In contrast with the results observed in CAT and CbE, the DHE activity remained very high in the biofilm samples. The trend of DHE in APW biofilm was similar to that observed for the substrate without earthworms.

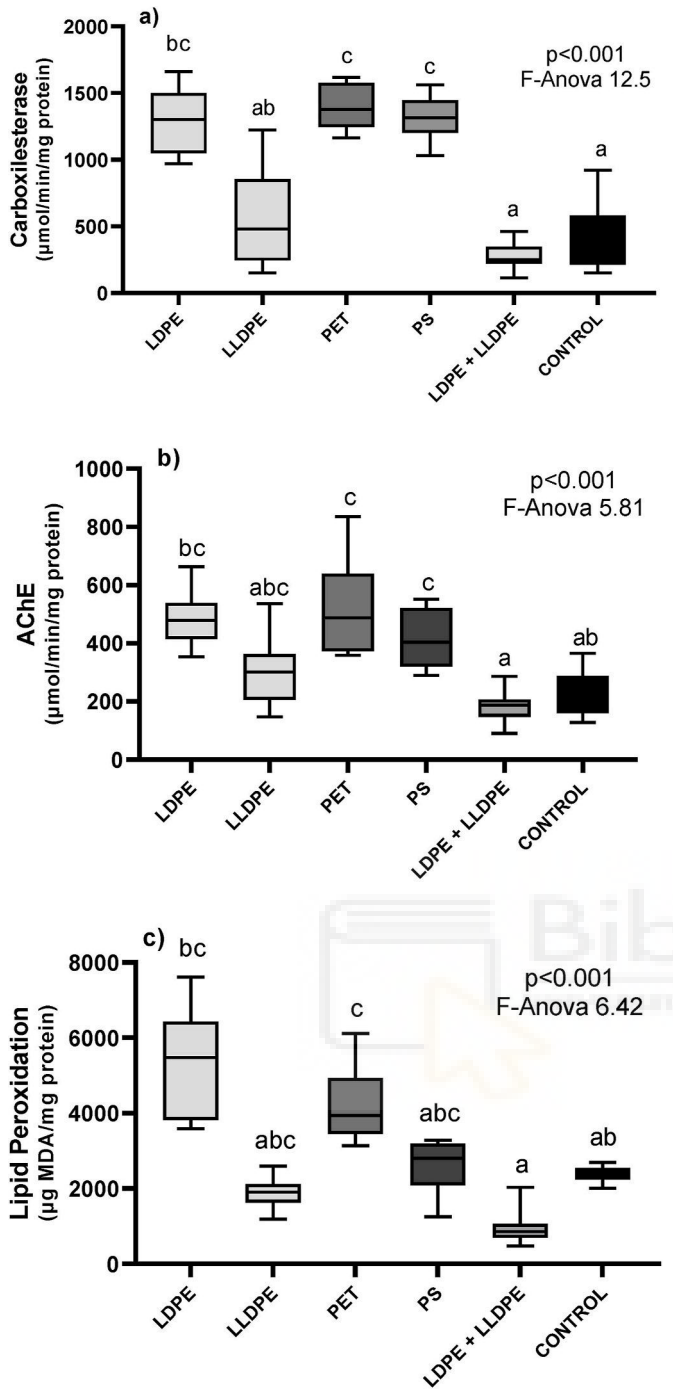

Fig. 3. Response of biomarkers: (a) carboxylesterase, (b) acetylcholinesterase (AChE) and (c) lipid peroxidation determined in *Eisenia fetida* tissue. Different letters in the box indicate significant statistical differences ($P < 0.05$).

3.3. Effects of AWP presence on Eisenia fetida: survival, body weight and response of biomarkers

The presence of APW in the feedstock seemed to induce a decrease in the *E. fetida* survival compared with the control treatment (Fig. 2). The treatments with LLDPE and the mixture LDPE + LLDPE produced the lowest value of *E. fetida* survival at the end of the exposure assay. In the PS, PET and LDPE treatments, statistical effects were detected, but with a decrease of the survival rate of less than 10% compared to the control treatment. This affectation observed in epigeic earthworms when they are exposed to APW material under vermicomposting conditions has been described as a set of biotic factors, which affects various physiological processes, such as respiration rate, reproduction rate, feeding rate and burrowing activity (Yadav et al., 2011). Concerning to weight variation, *E. fetida* exhibited a higher susceptibility to negative morphological effects by exposure to LLDPE and/or LDPE + LLDPE plastic treatments, obtaining similar values of negative weight variation. In the rest of treatments (PET, PS and LDPE), a similar trend was observed, although with minor differences compared with the control treatment. The substrate corresponding to the control treatment was able to maintain a higher density of earthworms with less mortality, also founding an enhancement of the body weight of *E. fetida* compared with the results observed in the substrate with plastic. This demonstrates that the nutrient capacity of the substrate material was not a limiting factor for *E. fetida* development, in the experimental bioassay. Thus, these results can indicate that the loss of weight in the treatments with the plastic presence could be induced by stress in the earthworm physiological activity.

Concerning the biomarkers studied in the *E. fetida* tissue, two different stress responses were observed in the *E. fetida* tissue by exposure to APW materials (Fig. 3). The treatments with the APWs including LLDPE in the backbone polymer formation (LLDPE and LLDPE + LDPE) were the only treatments that showed the mean value of CbE activity close to the control treatment. On the other hand, the highest CbE mean values were observed in the treatments where *E. fetida* was exposed to PS and PET. Lackmann et al. (2022) studied the CbE as a biotransformation enzyme involved in the xenobiotic metabolism, but reported that overall change in *E. fetida* was not significantly affected by the microplastic (Polystyrene-HBCD) exposure in soil for 28 days. Thus, the results obtained seem to suggest a different molecular mechanism underlying the earthworm response to the different APW tested. Chen et al. (2020) also studied the effect of different types of microplastic and reported a varying mode of action depending on the plastic type, but also the shape, size and potential influence of additives. Our results might indicate that the chemical nature of the plastic polymer is also determinant in the pathway followed by this type of esterase.

Acetylcholinesterase (AChE) is a biomarker of neuro-toxicity, which degrades acetylcholine to remove the neurotoxic effects of pollutants in many species (Zhang et al., 2020). The presence of APW materials produced an interaction of CbE and AChE of *E. fetida* in a similar way. AChE activity in *E. fetida* exposed to LDPE, LLDPE, PET, PS, significantly increase compared to the control, showing also the highest values with the exposure to PS and PET as it has been previously reported for CbE activity (Fig. 3). However, the individuals of *E. fetida* in LDPE + LLDPE and LDPE treatments showed similar AChE mean values than individuals in the control treatment. Previous studies have also shown an increase of AChE when *E. fetida* has been exposed to plastic materials, which could be due to the presence of plastic produces a stimulation of the neurotoxicity response in *E. fetida* to have a specific regulatory effect on neurotoxins (Chen et al., 2020; Zhong et al., 2021; Zhang et al., 2020). Thus, Chen et al. (2020) found an increase in AChE in *E. fetida* exposed for 21 days and 28 days at 1.0–1.5 g/kg of LDPE in soil. Zhong et al. (2021) also reported increases in AChE in a study about the effect of microplastics in sludge on the vermicomposting process.

Concerning to lipid peroxidation (Fig. 3), the presence of LDPE and PET induced the highest values of lipid peroxidation, compared to the control treatment without APW exposure. Recent studies have demonstrated that microplastics with a wide variety of chemical composition can cause in terrestrial organism such as ciliates, collembolans and earthworms skin damage, tissue lacerations, immunity disturbing and neurotoxicity with subsequent increase in lipid peroxidation activity (Sarker et al., 2020; Wang et al., 2020). Some studies have even shown that exposure to MPs (HDPE, PP and LDPE) with size less than 300 µm by E. *fetida* (Chen et al., 2020; Jiang et al., 2020) led to the occurrence of clear inflammatory processes, between the gut epithelium and the chloragogeneous tissue, sometimes with the development of fibrosis and congestion (Rodríguez-Seijo., 2018).

Table 2
Effect of the different factors (AWP presence, particle size and UV pre-treatment) on the main physico-chemical and chemical characteristics of the vermicompost obtained.

	pH	EC (dS m^{-1})	OM (%)	TOC (%)	TN (%)	P (%)	K (%)	WSC (%)	C_{FA} (%)	C_{HA} (%)
AWP presence										
Presence of AWP	7.6 b	4.2 a	54.5 b	26.8 a	2.3 a	0.7 a	1.2 a	8.6 a	2.5 a	3.3 a
Absence of AWP	7.5 a	5.1 b	52.0 a	25.3 a	2.4 b	0.8 a	1.2 a	8.2 a	2.9 b	3.8 a
F-Anova	*	***	***	*ns*	*	*ns*	*ns*	*ns*	*	*ns*
Particle size										
Film debris (mesoplastic)	7.6 a	4.0 a	54.1 a	24.5 a	2.4 a	0.7 a	1.0 a	8.3 a	2.4 a	3.1 a
Microplastic	7.7 b	4.4 b	54.8 a	29.2 b	2.3 a	0.7 a	1.3 b	9.0 b	2.5 a	3.5 b
F-Anova	*	***	*ns*	***	*ns*	*ns*	***	*	*ns*	*
Pre-treatment										
No pre-treatment	7.6 a	4.2 a	54.5 a	25.9 a	2.3 a	0.7 a	1.1 a	8.5 a	2.5 a	3.2 a
UVC – 250h	7.8 b	4.5 b	54.8 a	31.2 b	2.4 a	0.8 a	1.4 b	9.3 a	2.6 a	4.0 b
F-Anova	*	*	*ns*	***	*ns*	*ns*	***	*ns*	*ns*	**

EC: Electrical conductivity. TOC: Total organic carbon. OM: Total organic matter. WSC: water-soluble carbon, C_{FA}: fulvic acid-like C; C_{HA}: humic acid-like C. n.s.: not significant $P > 0.05$; *, **, ***: significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Average values in a column followed by the same letter are not significantly different at $P < 0.05$ (Tukey-b post-hoc test).

Thus, the plastic with less effect on the response of the biomarkers studied in the *E. fetida* tissue was the mixture of LDPE + LLDPE in all the cases, with values statistically similar to those obtained in the control treatment without plastic. This fact could be related to the production of these plastics (LDPE + LLDPE), since the joint extrusion process of both generates a new polymer. This mixture would have had a different crystallinity than the individual plastics, which could change the possible release of toxic substances that would alter enzymatic activity, such as those of CbE and AChE, as well as lipid peroxidation.

3.4. *Effects of the plastic materials on vermicompost properties and on E. fetida tissue biomarkers: AWP presence, particle size and UV pre-treatment*

All the plastic factors studied (presence, particle size and pre-treatment) had a significant effect on the physico-chemical parameters (pH and electrical conductivity) in all the scenarios studied (with and without earthworms) (Table 2). However, the effect on the rest of chemical parameters was different depending on the factor considered, with a more similar behaviour in case of the particle size and the UV-pre-treatment, both affecting the contents of total organic C, K and humic acid-like C. This similarity could be explained due to the changes produced at physical and/or chemical level, which can induce changes in the polymeric structure of the AWP materials, becoming the bulk polymer more available for biological attack (Shah et al., 2008; Urbanek et al., 2021).

Slight differences were found for the enzyme activities considered depending on the AWP particle size (film debris or microplastic-MP) and the application or not to the AWP materials of the UV pre-treatment (Fig. S1). For both factors, the only enzyme activities affected were CbE and DHE, not observing any significant effect on the catalase activity. Moreover, film debris seems to induce a lower inhibition in CbE and DHE activity than MP. Likewise, the pre-treatment of the plastic materials with UV-C during 250 h produced a lower activity in these enzymes. The lower enzyme activities observed for the AWP with smaller size (MP) or pre-treated with UV could be related with a lower biological activity oxidation of the organic matter content in the substrate due to the potential release of toxic compounds derived from the plastic materials when they are physically and/or chemically modified. Thus, the significant differences found in the enzymatic activities related to the size and/or composition changes can be an evidence of the presence of derived compounds from plastic material that can induce adverse effects. On the other hand, the particle size or the UV pre-treatment seem not to have a significant effect on the biomarkers studied (carboxylesterase, AChE and lipid peroxidation) in the *E. fetida* tissue (Fig. S2). Several studies have reported that the effects of MPs on terrestrial organism is closely related with different physico-chemical properties of polymer such as shape, size, types and additives (Chen et al., 2020; Lambert et al., 2017). However, the results obtained in this study are not in accordance with that reported in previous studies with earthworms in soil.

4. Conclusions

The presence of agricultural plastic waste-AWP (microplastic and film debris) influenced the final characteristics of the vermicomposts obtained, concretely organic matter and NPK contents, especially in the presence of PET and PS. The exposure to the AWP materials produced in *E. fetida* a higher susceptibility to negative morphological effects, observing the lowest value of survival and the highest loss of weight at the end of the exposure assay, especially with the treatments with LLDPE and LDPE + LLDPE. This fact was also reflected in the signs of oxidative stress and neurotoxicity observed in *E. fetida* related with APW exposure, especially with MP or AWP pre-treated with UV, which seemed to trigger a biochemical response that was not observed in the biomarkers response. However, more research is necessary to better understand the mechanism involved in the detoxification response system of *E. fetida*.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122027.

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Unlocking the biotechnological and ecotoxicological perspectives of microplastic degradation by means *Eiseinia fetida* inoculated with polymer degrading capabilities microorganism consortia.

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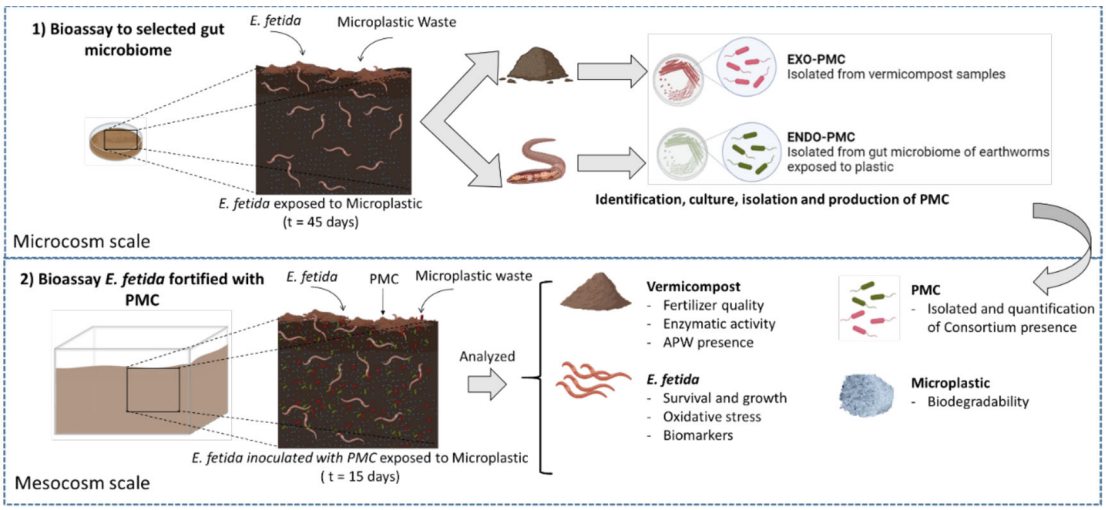
Graphical abstract



Abstract

The use of organic amendments (compost or fresh waste) polluted with microplastic lead to impact the quality of agricultural soil. In this study, the Microbiome isolated from *Eisenia fétida* gut content previously exposed to presence of different proportion of plastic (LDPE-2.5% and 5%) were tested as probiotics of earthworms during vermicomposting of microplastic-spiked biowaste (12,500 mg/kg) in mesocosm-scale trials (LDPE and Mix Plastic composed by LDPE+LLDPE+PS+PET). The survival and weight variation of earthworms were measured as well as the main enzymatic biomarkers related with oxidative stress. Culture selective medium and 16S *rRNA* amplification techniques were used in order to trace the presence of inoculum in the vermicompost obtained and gut content of *Eisenia*. Finally, the efficiency of the plastic degradation process was tested with thermogravimetry (TGA-MS) coupled with mass spectrometry technique. The result obtained indicate an improvement in survival rate and body weight maintenance of earthworms exposed to plastic in presence of consortium inoculum. Additionally, a reduction in oxidative stress response were observed in earthworms with inoculated consortia. Respect to vermicompost obtained significant variation in NPK content, particularly when applying ENDO and EXO PMC inoculum were determined. The

biodegradability measured showed a low performance for LDPE during the 30 days of duration of Bioassay.

Keywords: ecotoxicology, microplastic biodegradation, earthworm, probiotics, agricultural plastic waste

Environmental implication

The authors confirm its significant environmental implication, whose objective is to evaluate the performance of gut microbiome of *Eisenia fetida* isolated from earthworms exposed to plastic materials in obtain earthworms with improve capacities to improve the tolerance and performance of biodegradation rate of plastic material during vermicompoting of plastic-spiked biowaste.

1. Introduction

Microplastic (MP) contamination of the terrestrial ecosystem is a growing environmental concern. Agro-industrial, agricultural and livestock processes generate a large amount of plastic waste. Both, primary and secondary microplastics (the latter are formed through the degradation and disintegration of larger plastic pieces; Cole et al., 2011; Canesi et al., 2016) influence the ecology, health, and function of soils, with consequences to multiple ecosystems. The widespread of use of non-degradable plastic in agriculture is attributed to a range of characteristics of this polymer that allow improve the management, productivity and quality of the agricultural system. In agroecosystems, plastic entry may occur through various pathways, with the most common including: a) the use and incorporation of plastic mulch films to improve plant growth and reduce moisture loss (Huang et al., 2020; Sun et al., 2020; R. Qi et al., 2020); b) the addition of municipally-derived organic fertilizers, digestates, or compost (Watteau et al., 2018); (c) the application of biosolids (van den Berg et al., 2020); (d) the accumulation of slow- release fertilizer coatings (Katsumi et al., 2021) and (e) atmospheric deposition (Allen et al., 2019) (f) irrigation from polluted sources (Bläsing and Amelung, 2018) or because of soil biota and mesofauna or animal activities (Rilling et al .,2017).

Previous studies have investigated the environmental impact of microplastic on soil mesofauna. Generally, soil fauna, including earthworms, snails, collembolans, and nematodes, have all been shown to be negatively impacted by film debris or MP (Rodríguez-Seijo et al., 2018; Zhu et al., 2018; Ju et al., 2019; Song et al., 2019; Kim et al., 2020; Selonen et al., 2020; Sáez et al.,2022). For instance, exposure to microplastics leads to a wide range of physiological alterations, mainly/ primarily to the digestive, reproductive or immune systems (Yadav et al., 2011; Rodríguez-Seijo et al., 2018; Lahive et al.,2019; Sáez et al.,2022).

In the biowaste treatment, it should be noted that the potential effects of the presence of plastics remain largely unexplored (de Souza Machado et al., 2019). There is still not enough research in

this regard, with practically no studies on the effect generated by exposure to plastic material in earthworms or the effect on compost fertilizer quality of the product obtained. However, MP contamination of organic amendments in agriculture should also be considered since these pollutants may have a wide range of adverse effects on agroecosystems (Martin de la Fuente et al., 2022). Specifically, compost is known to be a potential source of MPs in agricultural soils (Ruggero et al., 2020) due to incorrect or incomplete plastic waste disposal (Braun et al., 2021).

Several studies have reported about enzymes capable of degrading plastic debris including cutinase, laccase, protease, esterase, and urease, and accountable microbial species as Bacillus, Streptococcus, Pseudomonas, Aspergillus (Inderthal et al., 2021; Ara et al., 2023). Nevertheless, the performance achieved by Pure microbial cultures in degradation rate is very low under controlled lab-scale conditions, suggesting that the plastic biodegradation require the participation of wide range of metabolic routes linked to use of cu-culture microbiome (Carpena-Istan et al., 2024)). According with previous research, vermicomposting treatment could accelerate plastic degradation throughout the creation of two microhabitats with great enzymatic and microbial activity (Sánchez-Hernández et al.,2020). One of these habitats correspond with cast-associated processes with a vast variety of decomposer microorganism that could participate in the plastic biodegradation and the other habitat related with gut-associated processes where biochemical transformations are mediated by enzymes released to the luminal environment by both symbiont microorganisms and the earthworm gut epithelium (Soobhany et al., 2019).

Commonly used methodologies to assess the plastic degradation in compost are based on spectroscopic analysis (such as nuclear magnetic resonance, infrared spectrometry, etc), the measurement of CO_2 production and release or visual inspection (colour changes, surface roughness, surface morphology). However, these techniques present challenges in sample preparation, have high limits of quantification, and lack accuracy. In the recent year, research efforts have aimed to establish successful methodologies to determine MP in organic amendments

100 or biowaste. Nevertheless, quantifying MP in heterogenous solid matrix involves interference of the
101 bulk matrix that must be removing (Martín de la Fuente et al., 2022). Various thermal
102 methodologies have been applied to determine MP. Blancho et al 2021 reported the application
103 thermal extraction desorption gas chromatography mass spectrometry (TED-GC-MS) and pyrolysis
104 gas chromatography mass spectrometry (Py- GC-MS) to determine MPs. However, the laborious
105 sample processing steps, the minimal amount of sample required and the difficult to interpret the
106 data make this technique unavailable (Peñalver et al., 2020, Bitter and Lackner 2021) One
107 successful thermal methodology reported to determine MP in organic matrix is based on
108 Thermogravimetry coupled with mass spectrometry (Martin de la Fuente et al.,2022). Additionally,
109 this thermal methodology has demonstrated to be quick, cost-effective and easier to handling the
110 samples.
111 Based on above, this work aimed to study the potential of *Eisenia fetida* gut microbiome identified
112 and isolated from earthworms previously exposed to plastic material in produce earthworms with
113 fortified capacities. For this, a mesocosm scale bioassay with inoculated consortia was carried out in
114 order to simulate the vermicomposting treatment of plastic-spiked biowaste (LDPE and Mix plastic,
115 composed by 32.5% LDPE + 32.5% LLDPE + 20% PS + 15% PET) for 30 days. During the
116 vermicomposting assay, the survival and weight variation of *Eisenia fetida* were determined. At the
117 end of bioassay, the main biomarkers involved in metabolic and oxidative stress in tissue of
118 earthworms as well as the physico-chemical characteristics and potential enzymatic activities in
119 vermicompost obtained were measured. The evolution and trace of inoculum consortia were also
120 tested by Culture selective medium and 16S *rRNA* amplification. Finally in order to determine the
121 performance of this biotechnological approach in biodegradation of plastic material measured of
122 TGA-MS were caried out.

123 ***2. Material and methods***

124 ***2.1 Isolation, characterization and selection of endogenous probiotics from Eisenia fetida***
125 ***exposed to plastic-spiked biowaste.***

To promote the presence of plastic-degrading microorganisms in the digestive tract of *Eisenia fetida* specimens, mature compost (standard diet of *E. fetida* earthworms) was artificially contaminated with two different doses of low-density polyethylene (LDPE):2.5% w/w and 5% w/w. The experiment was conducted in 18 Petri plates (15 cm Ø), each containing 25 *E. fetida* adult citellated specimens and 80 g of compost. Petri plates were incubated at 20ºC ± 2 in total darkness for 45 days. Each plastic type was tested in triplicates (n=3). In addition, specimens were fed a plastic-free diet as controls and individuals from each plastic-feeding condition underwent a fasting regimen to ensure the complete emptying of digestive contents after the incubation period.

To isolate endogenous plastic microbial consortia (ENDO-PMC) from the digestive tract of *E. fetida*, specimens were surface sterilized (to prevent cross-contamination with epithelial microbiota) by sequential immersion in sterilizing solutions. Initially, earthworms were rinsed in sterile water (5 sec) and sterilized using 95% (vol/vol) ethanol (5 sec), 0.5% NaClO (2 min), 70% vol/vol ethanol (2 min), followed by three rinses (1 min each) in sterile distilled water. The sterilized specimens were then placed in Eppendorf tubes and homogenized utilizing a pottermiller (Heidolph Company) under sterile conditions. Subsequently, 10-fold serial dilutions were performed, and 100 µL aliquots were plated on both APHA and Rose Bengal (RB) (Panreac) culture media for the enumeration of general bacteria and fungi, respectively. Additionally, Remazol Brilliant Blue Reagent (RBBR) culture media was used to quantify microorganisms with ligninase capability, an enzymatic activity related to plastic biodegradation The composition of the RBBR medium includes (per liter): 10 g glucose, 5 g peptone, 2 g yeast extract, 200 mg RBBR dye (Sigma), 20 g agar, and 70 mL of trace element solution (Martinez-Gallardo et al., 2020). Petri plates were incubated for 24 hours for bacteria and 72 hours for fungi at 30 °C. Colonies that stood out as the predominant ones in the test samples were chosen for utilization as probiotics in the following assay. Strains exhibiting easy culturing conditions (maintenance of viability after preservation), abundance in the overall population, and non-pathogenicity were selected, isolated and taxonomically identified.

In order to taxonomically identify the selected strains, pure cultures of each strain were subjected to genomic DNA extraction utilizing a heat shock procedure:(5 min /96 ºC and 5 min /4 ºC). Molecular identification through 16S rRNA gene amplification was executed utilizing universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907R (5′-GGTTACCTTGTTACGACTT-3′) (Isik et al., 2014). The PCR thermal conditions included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 30 seconds, and a single final step of 72 °C for 10 minutes. Amplicon size was verified through electrophoresis in 1% agarose gel stained with GelRed (Biotium Inc., Hayward, CA, United States). Finally, the amplicons purification was performed using E.Z.N.A cycle pure kit (Omega-biotek, Norcross, GA, United States) and sequencing was performed in the Sequencing Service at the University of Almeria (SSA) through the dideoxynucleotide cycle sequencing method on an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, United States).

In addition, two previously identified bacterial strains belonging to the collection of research group BIO-175 at the University of Almería were utilized as exogenous plastic microbial consortia (EXO-PMC) to enhance the plastic biodegradation in the substrate provided to the earthworms. This selection was made according to comprehensive research findings. *Pseudomonas putida* REBP7 was isolated through a sequential enrichment experiment, employing linear low-density polyethylene (LLDPE) in powder format (<500 μm) as the sole carbon source (Salinas et al., 2023). This strain showed enzymatic activities related to plastic biodegradation such as cutinases and polyphenol oxidases, as well as the ability to decolorize RBBR (Remazol Brilliant Blue Dye). On the other hand, *Bacillus subtilis* RBM2 was isolated from compost samples of composted vegetable residues and ultimately selected due to its notable enzymatic capabilities, including lacases and lipases, involved in plastic degradation (Jurado et al., 2014). These microorganisms exhibited high metabolic versatility and complementary enzymatic activities, rendering them suitable for consortium assembly. The diverse metabolic capabilities and cooperative enzyme functions

observed in these microorganisms contribute to their potential effectiveness in degrading plastics through synergistic interactions.

2.2 Experimental set up: Eisenia fetida fortified with plastic degrading capacities consortia during vermicomposting in mesocosm scale trials

The second bioassay consisted in mesocosm bioassay whereby 100 adult of *E. fetida* were incubated for 30 days in containers (4L) filled with 640 g f.w. of feedstock adjusted with distilled water to 70% moisture content. Then, 8 g of AWP material was added per replicate (12,500 mg/kg). The incubation containers were kept under same conditions that previous trials (20° ± 2 °C and darkness). At the end of exposure time (30 days), survival and body weight variation were determined. For this, earthworms were carefully extracted from the container (4L) of each replicate by hand sorting, counted for survival, weighted, and recorded to obtain the mean body weight of each treatment. During the exposure time of the bioassay, when mortality was observed, the earthworms were immediately removed from the container. In addition, at the end of the bioassay, the final materials obtained were treated in the same way as the previous bioassay. Two different Plastic materials, LDPE and Mix-plastic (32.5% LDPE + 32.5% LLDPE + 20% PS + 15% PET) were tested at microplastic particle size.

This mesocosm assay was conducted to assess the persistence of ENDO and EXO-PMC in the *E. fetida* gut and their effectiveness in enhancing the earthworms tolerance to plastic exposure. Initially, the inoculum was produced from the probiotics isolated in the previous phase at a laboratory scale. Pure cultures were incubated overnight at 30 °C in 100 mL of nutritive broth (NB) (Panreac) and centrifuged in 250 mL Nalgene bottles at 5000 rpm for 5 min using an Eppendorf centrifuge 5810 R to obtain concentrated biomass. The biomass was frozen at -80 °C and then lyophilized using skimmed milk (Scharlau) as a cryoprotectant in a Telstar LyoQuest freeze drier (Azbil Telstar Technologies SLU, Spain) at -60 °C for 24 h under a pressure of 0.6 mBar. The bacterial load was assessed and adjusted through 10-fold serial dilutions in sterile distilled water

until reaching 10^7 CFU/g. ENDO-PMC suspension was sprayed onto the feedstock to fortify the *E. fetida* gut microbiome. Furthermore, to enhance plastic biodegradation in the *E. fetida* feedstock, EXO-PMC was inoculated in the *E. fetida* diet and the bacterial load was determined as previously explained. Additionally, intending to test the synergistic effect of both EXO+ENDO-PMC in plastic biodegradation, a third experimental condition was assayed by spraying a combination of both consortia in *E. fetida* feedstock. To assess the persistence of the inoculum in both the feedstock and the digestive tract, these three experimental conditions were tested in both conditions.

2.2.1 Surveillance and measurement of E. fetida ENDO and EXO-PMC

The extraction and homogenization of *E. fetida* digestive tracts were carried out following the procedures outlined in the preceding Section 2.1 to detect the presence of ENDO-PMC. Additionally, substrate samples were collected at the end of the incubation period to assess the presence of EXO-PMC. Subsequently, 10-fold serial dilutions were prepared using sterile saline solution (0.9% w/v) and 100 µL samples were plated in triplicates in Cetrimide Agar (Scharlab) for *Pseudomonas* genus detection and Chromoselect Agar Bacillus (Sigma) for *Bacillus* genus detection. Counts were performed after incubation at 25°C for 72 hours.

2.3 Biomarkers of Earthworms

At the end of mesocosm bioassay (30 days), the earthworms from each replicate were carefully recovered and weighed to obtain post-incubation body weight. Subsequently, earthworms were placed on Petri dishes, and after 24 h of starvation, they were euthanized by freezing. The dissection process, conducted using a stereomicroscope, involved rising the muscle (body wall) tissues with distilled water to remove any rest of coelomic fluid. The rinsed tissues were collected and promptly frozen at -80ºC. Previous to analysis, the muscle tissue was homogenized in ice-cold buffer (pH = 7.4) made of 25mMsucrose, 20mM Tris-HCl buffer, and 1mM EDTA by milling with potter (Ika T18 digital). The homogenates were centrifuged at 9000 g for 20 min at 4◦C to obtain the post-mitochondrial fraction. For the determination of Biomarkers measured in muscle tissue of

earthworms, Carboxylesterase (CbE), Acetylcolinesterase (AChE) and lipid peroxidation were assessing following the procedure described by Sáez et al.,2022. The glutathione-dependent antioxidant enzymes, GSH (reduced glutathione) and GSSG (oxidized glutathione) were determined following the method described by Rahman et al., 2006. For this, 100 µl of homogenised tissue of earthworms were firstly deproteinized by adding 100 µl of sulfosalicylic acid (0.6 %). Protein removal was achieved by centrifugation, and 50 µl the acid supernatant mixed with 125 µl of 1:1 (v:v) DTNB (5.5′-dithiobis-2-dinitrobenzoic acid) and glutathione reductase. Finally, 50 µl of NADPH was added and changes immediately monitored at 412 nm. For GSSG measurement, 50 µl of 2-Vinylpyridine were added and incubated for 1 hour to derivatize GSH, neutralized with triethanolamine and finally the sample were treated with same procedure as GSH determination.

In order to standardize and asses all biomarkers response of earthworms to different kind of agricultural plastic and integrated them in a single value, we used the "Integrated Biomarkers Response" (IBRv2) described by Sánchez et al., 2013, which was calculated as follow (eq1):

$$IBRv2 = \sum_{i=1}^{n} |A_i| \quad (1)$$

Where A_i was obtained from the equation (eq2), which calculates the deviation indices of each plastic treatment regarding to reference value (no plastic treatment)

$$A_i = \frac{Y_i}{\sigma_{ref}} \quad (2)$$

Where Y_i represent the log transformation applied to general mean to reduce the variance and σ_{ref} is the standard deviation of reference value. Additionally, individual values were plotted in a star plot to identify inhibition (negative Ai in the Star plot below the reference level) or induction response (positive Ai or area above the reference level) for each biomarker, this approach facilitated the identification of which biomarker had a stronger impact on the IBRv2sum value. The reference value used was obtained from the control treatment without plastic or inoculum addition.

2.4 Phsysico-chemical analytical methods

The physicochemical parameters in vermicompost samples previously dried at 105ºC were determined in 105 as following described; electrical conductivity (EC) and pH were measured in a 1:10 water extract (w/v). Moisture content was determined after drying to constant weight at 105 °C for 24h. Organic matter (OM) content in compost samples by loss on ignition at 430 °C for 24 h. Total organic carbon (TOC) and total nitrogen (TN) were determined by burning the samples at 1020 °C in an automatic elemental micro-analyzer (*EuroVector Elemental Analyzer. Milano. Italy*). After (HNO_3/H_2O) (1:1 v/v) microwave (*CEM. mod. MARS ONE*) assisted digestion; macronutrient as P and K and other micronutrient were determined by ICP-OES. Ç

2.5 TGA-MS procedure to determine the biodegradability efficiency of microplastic

In order to assess the biodegradation performance of biotechnological process stablished used different consortium inoculated in earthworms a TGA/DSC 1 HT thermogravimetric analyzer (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) was employed for the thermal decomposition of the samples in a nitrogen atmosphere (50 mL min^{-1}). The temperature was increased from 30 to 800 °C at 10 °C min^{-1}. All the TGA analyses were corrected used blank curves. The TGA system were linked to a Balzers Thermostat mass spectrometer (Pfeiffer Vacuum, Asslar, Germany) for evolved gas analysis, utilizing a QMS 200 M3 Quadrupole mass spectrometer. The mass spectrometer detector, operated in Single Ion Monitoring Mode (SIM), measured the ions *m/z* 56 for PE and *m/z* 104 for PS for quantification purposes. A dwell time of sixty seconds was employed for every ion, with a cathode voltage of 65 V. Approximately 10 mg of the sample were accurately weighed and then placed in 70 µL alumina crucibles without lids. Data analysis was performed with IBM SPSS 28 Statistics software. A seven-point calibration curve was constructed, with each concentration analyzed in duplicate. This sample was spiked with PE and PS ranging from 2 to 2000 mg kg^{-1} and from 0.2 to 200 mg kg^{-1}, respectively, for calibration standards. The quantification of the samples was carried out by integrating the area under the MS curves for the 56 and 104 *m/z*

ions for PE and PS, respectively, within the 350 to 550 ◦C temperature range. Finally, the analyses described above were carried out each one in triplicate.

2.6 Statistical analysis

The IBM SPSS Statics V.28 software package was used for the statistical analyses. For survival, weight variation, physicochemical parameters and enzymatic biomarkers the multivariate general linear model (GLM) were used to assess the significance of difference among the results obtained, taking into account the effect of main variables (Type of plastic material and Type of Inoculum). LSD test were also conducted with Tukey-b and Duncan as post hoc test. The normality of the distribution and homogeneity of the variances were checked using the Shapiro-Wilk and Levene test, respectively, before ANOVA. For the Microbiological measured, data obtained from the experiments were subjected to analysis of variance (ANOVA; $p < 0.05$), followed by Fisher's LSD (Least Significant Difference) test to compare mean values among different treatments and ascertain significant differences. The results of the statistical analysis are visually represented in figures, with error bars indicating the LSD interval. The statistical procedures were performed using Statgraphics Centurion XIX version 19.4.01 (Stat-Point, Inc.). Data were graphically represented using Microsoft Office Excel 365.

3. Result and discussion

3.1 Selection of plastic degrading microorganisms for fortification of E.fetida gut microbiome

Earthworm specimens subjected to the abovementioned experimental conditions were homogenized and plated in several culture media. Selection of probiotics was founded on the criteria mentioned in Section 2.1. Finally, nine strains were selected and molecularly identified as illustrated in Table 1.

300 ***Table 1.***. Taxonomic identity and selection of probiotics isolated from *E.fetida* gut microbiome

ID strain	Plastic dose (%)	Taxonomy	Reference taxonomy	Homology (%)	Base Pair
S-ALME2 - B1	5	*Bacillus altitudinis*	MT598007.1	99.9	896
S-ALME2 - B2	5	*Bacillus licheniformis*	MT642945.1	100	896
S-ALME2 - B3	5	*Bacillus licheniformis*	MK583660.1	100	936
ALME2 - B1	5	*Pseudomonas putida*	JQ086574.1	99.9	884
ALME2 - B2	5	*Pseudomonas putida*	CP045551.1	99.9	907
ALME2 - B3	5	*Bacillus sonorensis*	KM817233.1	98.6	904
S-ALME1 - B1	2.5	*Bacillus pseudomycoides*	MH202942.1	98.2	903
ALME1 - B1	2.5	*Bacillus mycoides*	MW756955.1	98.0	914
ALME1 - B3	2.5	*Bacillus licheniformis*	MT642946.1	100	693

301

302 Identification revealed the presence of two well-known genera in the literature recognised for their
303 ability to degrade plastics (Vimala and Methew, 2016; Kyaw et al., 2012). Specifically, 22% of the
304 identified strains belong to the genus *Pseudomonas*, while the majority 78% are classified under the
305 genus *Bacillus*.

306 In particular*, Pseudomonas putida* has been extensively studied for its remarkable capability as a
307 plastic degrader. This strain is effective not only in reducing plastic weight but also in achieving
308 complete mineralization of highly recalcitrant plastics such as LDPE. The evidence of this
309 biodegradation has been substantiated by the observation of craters on the plastic surface using
310 scanning electron microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR) (Pathak
311 2023). Additionally, other strains of *Pseudomonas putida*, selected in this study as EXO-PMC from
312 a plastic enrichment culture, further support this evidence (Salinas et al., 2023).

313 As for *Bacillus* genus, specific strains have gained significant attention in the scientific community
314 due to their role in plastic biodegradation. In particular, *Bacillus licheniformis* and *Bacillus sonorensis*
315 stand as pivotal contributors to the overall effectiveness of the *Bacillus* genus in plastic degradation.
316 In the investigation conducted by Kučić Grgić et al. (2023), these two *Bacillus* species were identified
317 in activated sludge and compost, presenting themselves as promising candidates for inclusion in a
318 consortium to degrade LDPE films. An additional layer of significance is attributed to *Bacillus*

licheniformis, renowned for its role as a surfactant producer, driving the formation of biofilms —the crucial first step in plastic biodegradation. Interestingly, despite their plastic degradation capabilities, *Bacillus licheniformis* and *Bacillus sonorensis* also demonstrate the ability to produce polyhydroxyalkanoates (PHA) and polyhydroxybutyrates (PHB) - molecules employed in chemistry as monomers to produce bioplastics (Mittal et al., 2023; Kumar et al., 2013). The juxtaposition of plastic biodegradation and bioplastic production renders this strain particularly intriguing for implementation in circular economy strategies. Therefore, based on the result obtained, three strains were selected to constitute ENDO-PMC for the fortification of *E. fetida*: *Pseudomonas putida* ALME2-B1, *Bacillus sonorensis* ALME2-B3, and *Bacillus licheniformis* S-ALME2.

3.2 Effects of MP in characteristics of the vermicompost obtained

The data obtained in the determination of the main physicochemical parameters in the vermicompost inoculated with PMC are shown in table 2. Regarding pH, a slight decrease was observed in all treatments during the 30 days of incubation. The pH values at the end of the bioassay remained within the range suitable for worm activity and microorganism growth (5.5-8.5) (Yadav et al., 2011) in all treatments.

In terms of parameters indicating the degradation process of the worms, both the MOT content, TOC and C/N ratio show slight but significant differences, with the treatment with Mix-plastic showing the highest values of TOC and C/N ratio and MOT values. Middle values were obtained for vermicompost spiked with LDPE, but in all cases the differences with respect to the Control treatment are low. Observing the EC results, its value increases considerably in treatments with plastic presence, as well as the Control treatment. The increase in EC during the vermicomposting process has been reported by other authors (Khali and Sanaa, 2009; Fernández-Gómez et al., 2010). The increase in EC may be attributed to greater mineralization of organic matter, which released nutrient ions and soluble salts (Huang et al.,2017). Regarding the nutritional content of the obtained vermicompost, there is a slight but significant variation in NPK content, particularly when applying ENDO and EXO

PMC inoculum. This application notably increases the phosphorus (P) and potassium (K) content. Interestingly, the presence of plastic did not result in major differences, suggesting it does not significantly affect the nutritional composition of the vermicompost.

Table 2. Results of the Psychico chemical effects of microplastic presence depnding of PMC inoculated or type of plastic matrials

	pH	EC (dS m^{-1})	TOM (%)	TOC (%)	TN (%)	C/N	P (%)	K (%)
	T30 d	**T30 d**	**T30 d**	**T30 d**	**T30 d**	**T30 d**	**T30 d**	**T30d**
Type of AWP								
No AWP	8.06	4.28	37.2 a	22.9 a	1.97	11.6 a	0.53 c	0.88 b
LDPE	8.01	4.39	38.8 c	24.2 b	1.95	12.4 b	0.47 a	0.83 a
Mix-plastic	8.04	4.39	38.3 b	25.0 c	1.99	12.6 b	0.50 b	0.86 ab
F-anova	*ns*	*ns*	*66.2****	*25.7****	*ns*	*10.3****	*12.0****	*4.41**
Type of PMC								
No PMC	8.02	4.25	38.9 c	23.2 a	1.95	11.9	0.47 a	0.80 a
ENDO	8.02	4.37	39.3 d	25.0 c	2.01	12.5	0.53 b	0.92 b
EXO	8.05	4.39	37.7 b	24.3 bc	1.95	12.4	0.52 b	0.90 b
Mix c	8.05	4.41	36.4 a	23.6 ab	1.97	12.0	0.49 a	0.82 a
F-anova	*ns*	*ns*	*128****	*11.5****	*ns*	*ns*	*9.5****	*17.0****
Statistical significance								
Type of AWP	*ns*	*ns*	*****	*****	*ns*	*****	*****	*
Type of PMC	*ns*	*ns*	*****	*****	*ns*	*ns*	*****	*****
AWP X PMC	*	*****	*****	*****	*****	*ns*	*	*ns*

EC: Electrical conductivity, TOC: Total organic carbon, TOM: Total Organic matter. Mix-plastic (32.5% LDPE + 32.5% LLDPE + 20% PS + 15% PET). ns, *, **, *** indicate not significant, statistically significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively. Average values in a column followed by the same letter are not significantly at $P < 0.05$ (Tukeys -B and Dunacn post -hoc test).

3.3 Survival and Body weight evolution of Eisenia fetida

As previously detailed, three microorganism species were selected from the earthworms intestinal tract to evaluate their potential use as probiotics and were applied at a dose of 10^8 CFU/g on feedstock. The assessment of probiotics consortium inoculated feedstock (ENDO-PMC) revealed significant improvement in EF survival compared to the control. In EXO-PMC inoculated feedstock, no detrimental effect on survival were observed compared to the control treatment without microorganism inoculation. Notably, the Mix c (ENDO+EXO) showed the highest survival rate.

As illustrated in Fig 1, the inoculation with ENDO and EXO consortium significantly improved the survival, particularly in presence of LDPE, and specially for Mix plastic. These findings contrast from those reported in a study of vermicomposting of sewage sludge with presence of microplastic (PP and HDPE), where no significant effect on survival were found (Ragoobur et al., 2022). In concordance with this, Judy et al., 2019 in incubation of *Eisenia fetida* reported no significant differences in earthworm survival, growth or reproduction between the municipal organic waste amended soil controls and the municipal organic waste amended soil with microplastics (HDPE and PET) treatments. This corroborates the hypothesis of the improvement of *Eisenia fetida*, suggesting a synergetic effect in both: a) Gut-associated microorganism of earthworms (ENDO-PMC) with the subsequent improve in their physiological activity and b) inherent feedstock microorganism activity enhanced by EXO-PMC.

Regarding to body weight loss, overall, the inoculation with the different microbial consortia tested were able to maintain lower loss of weight of *E fetida* compared to control treatment, but without significant differences (table 3). The treatment with the lowest mean weight reduction corresponds with EXO-PMC. After 30 days of exposure (Fig 1), two treatments exhibited a weight reduction exceeding 20% (LDPE-No PMC and Mix Plastic-Mix C), which could be indicative of a harmful effect on metabolic processes. Statistical differences were observed for the inoculation with EXO-PMC, where lower weight reduction was noted in LDPE and Mix Plastic exposure treatments (-9.5% and -1.4%, respectively) compared to the No plastic treatment (-12.6%). While No PMC and Mix C also showed statistical differences, but no clear relationship could be established between this observed effect and the action of PMC.

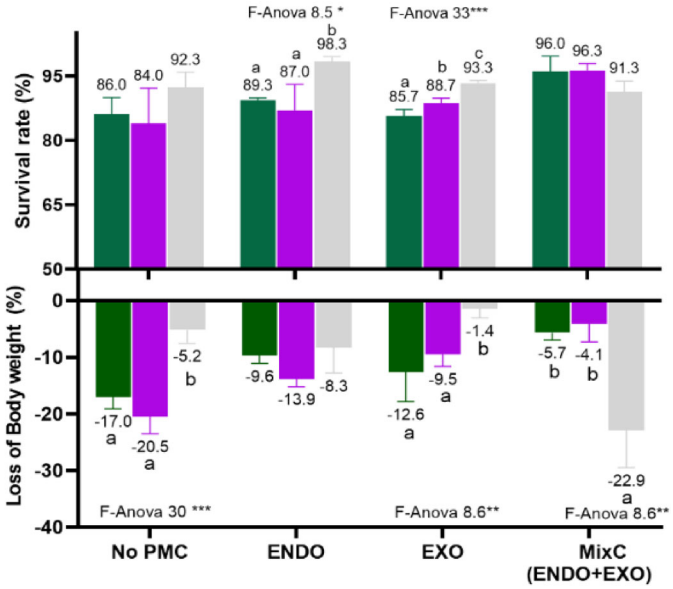


Figure 1. EF survival (%) and body weight variation (%) depending of the type of consortium and added plastic materials

Type of PMC	Survival (%)	Body weight variation (%)
No PMC	86.1 a	-14.5
ENDO	89.6 a	-10.6
EXO	85.9 a	-7.9
Mix c	95.9 b	-10.9
F-anova	*8.7***	*ns*

Table 3. Mean values of Survival and body weight variation (%) depending on the PMC consortia Inoculated.

3.4 Effect of MP presence and PMC inoculation on enzymatic activity of vermicompost

In the results obtained for the different enzymatic activities of the vermicompost (Table 4), we found significant differences in the activity of the dehydrogenase enzyme (DHE) according to the type of plastic material exposure after t30 d. As can be seen in Table 4, the presence of the MIX plastic led to a slight decrease in DHE activity in the vermicompost with respect to CONTROL, this being the only treatment showing significant differences. This decrease in DHE enzyme activity with MIX plastic could be due to a worse effect in rate of bioxidation of organic matter with consequent reduction in DHE activity. As regard to Catalase, also found significant differences from result of enzyme catalase activity according to the factor type of microbial consortium. The presence of the EXO inoculum led to a significant decrease in CAT activity in the vermicompost with respect to No PMC treatment. This decrease could be associated with a lower effective interaction between the inoculated EXO microorganism and the degradative activity in the earthworm gut. This result agrees with previous studies (Blesa Marco et al.,2023) where found an inhibitory effect in CAT activity during vermicomposting process exposure to PET and LDPE+LLDPE microplastic. In addition, Samal et al., 2023 reported a significantly decrease of hepatic catalase activity of human in presence of Polyethylene bags microplastics. In opposite trend, for carboxylesterase (CbE) activity, in general, the

three different inoculum consortia seemed to induce an increase in the values obtained. The exposure to LDPE and MIX plastic not induced higher change in CAT and CbE activity if compare with Control treatment.

Table 4. Values of the Enzyme activities measured in feedstock, before (t0d) and after vermicomposting process (t30d).

	CbE (nmol/h/g dry subsatrate)		**Catalase** (mmol H_2O_2/h/g dry substrate)		**Dehydrogenase** (nmol INTF/h/g dry substrate)	
	t0d	**t30 d**	**t0d**	**t30 d**	**t0d**	**t30 d**
Type of AWP						
No AWP	219 a	164	36.8 a	34.2 a	930 b	1035 b
LDPE	247 b	166	38.7 b	37.0 b	988 c	1037 b
Mix-plastic	220 a	163	35.8 a	34.4 a	843 a	944 a
F-anova	4.6*	ns	12.4***	5.9**	18.9***	11.3***
Type of PMC						
No PMC	282 b	152 a	37.1 b	38.5 c	1009 b	985
ENDO	199 a	167 b	34.9 a	35.4 b	833 a	1017
EXO	204 a	170 b	38.3 b	30.8 a	962 b	1002
Mix c	229 a	168 b	38.1 b	36.0 bc	878 a	1016
F-anova	20.2***	4.0*	11.2***	18.0***	17.0***	ns
Statistical significance						
Type of AWP	*	ns	***	**	***	***
Type of PMC	***	*	***	***	***	ns
AWP X PMC	6.8***	6.4***	11.9***	9.5***	14.0***	6.9***

CbE= carboxylesterase, Mix-plastic (32.5% LDPE + 32.5% LLDPE + 20% PS + 15% PET). ns, *, **, ***, indicate not significant, statically significant at P≤ 0.05, P≤0.01, P≤0.001, respectively. Average values (n=3) in a column followed by the same letter are not significantly different at P< 0.05 (Tukey's-b and Duncan post-hoc test).

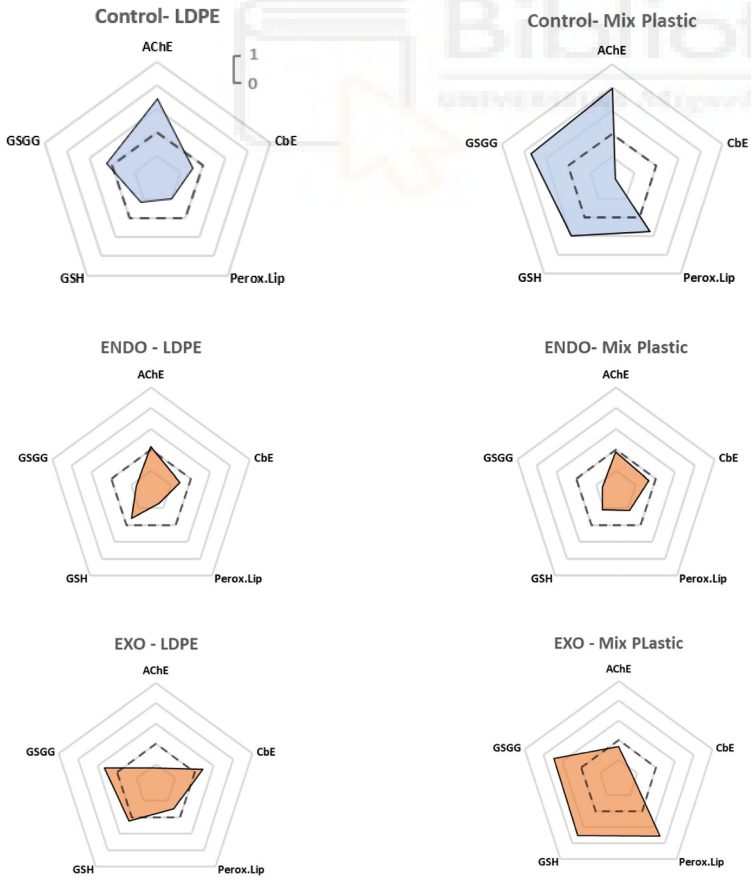
3.5 Ecotoxicological effect of MP in biomarkers response of Eisenia fetida

In this study, the IBRv2 index have been used to assess the integrate biomarkers responses. As shown in Fig 2., the index clearly showed that the exposure to Mix-plastic induced oxidative stress in earthworms, as evidenced by the high A_i scores for both GSGG and GSH activities in Control treatment with Mix-plastic. The exposure to Mix-plastic (Control-Mix plastic) also showed high A_i score of AChE and Lipid peroxidation both commonly related with neurotoxicity and cell damage, respectively. It is interesting to note the positive induction of several biomarkers was less evident by exposure to LDPE microplastics.

In previous studies that used the same species of earthworm (Sáez et al., 2022) the biomarkers response reported are comparable to obtained in control earthworms measured in this study. Therefore, we can assume that the data obtained in our study can be used as reference baseline values for IBRv2 index calculation. The result obtained in A_i score and their representation in star plots, revealed a differential behaviour depend on the PMC used. The inoculation with ENDO- PMC lead an inhibition effect in tissue

of earthworms in both LDPE and Mix plastic treatments at least for GSGG, GSH and lipid peroxidation. In the case of the earthworms with EXO-PMC addition, with the exposure to LDPE was observed a similar behaviour than Control-LDPE treatment without PMC inoculation used as baseline reference, except for AchE. In addition, for EXO-PMC, the exposure of MIX plastic led to change in GSGG, GSH and Lipid peroxidation in the same way that observed in Control-Mix-plastic. Finally, the response of biomarkers of earthworms to MIX c inoculation led to slight inhibition effect for AChE if compared with Control-Mix plastic.

The result obtained (Fig. x) show as the IBR v2 of plastic-exposed earthworms markedly increased from control treatment earthworms, varying from 3.88 for LDPE to 7.24 for PET, which indicate a general induction response in biomarkers determined. The most successful consortium for alleviate the oxidative stress induced by LDPE was the Mix c reached a final IBR v2 value of 2.59 and ENDO for the exposure to Mix plastic reached a final value of 3.54.



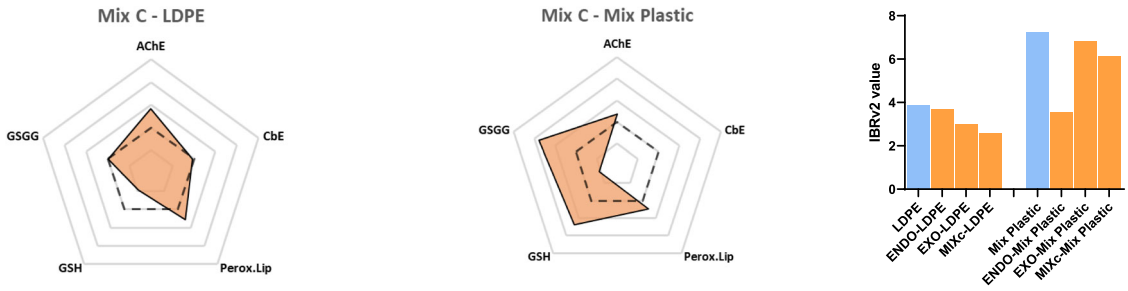


Figure 2. Star plots of the A_i score (deviation index) of biomarkers measured in muscle tissue of *Eisenia fetida*. Dotted lines in star plots indicate the reference values correspond to control earthworms (without addition of PMC or plastic). Bar plot shows the integrated biomarker response index (IBRv2) values calculated for each plastic type. MIXc= ENDO+EXO, AChE= acetylcholinesterase, GSH= reduced glutathione, GSGG = oxidized glutathione, Perox. Lip= lipid peroxidation, CbE= carboxylesterase.

3.6 Microbial community structure of E. fetida exposed to plastic and inoculated with ENDO and EXO consortium in mesocosm-scale

At the end of mesocosm-scale trials, surviving earthworms exposed to LDPE and Mix plastic in presence or absence of probiotics consortium (EXO and ENDO-PMC) were collected and their gut microbiome subjected to metataxonomic analysis. As illustrated in Fig 3., for Total Bacteria load in control treatment without inoculation of PMC no significant difference was found when earthworms were exposed to plastic (LDPE and Mix plastic), whilst in ENDO, EXO and Mix c (ENDO+EXO) the exposed to both plastic lead to a great Gut-microbiome load at the end of bioassay with statistical differences. Except for ENDO+EXO inoculated earthworms exposed to Mix plastic, the load of *Pseudomonas spp* in gut-microbiome of earthworms exposed to LDPE and MIX-plastic also became significant higher if compared with Earthworms without inoculum application. Finally, the presence of LDPE induced the great extent the increase in abundance of the *Bacillus spp*. In the case of exposure to plastic mixture, a significant increase was also observed when compared to the control, although this increase effect was less evident.

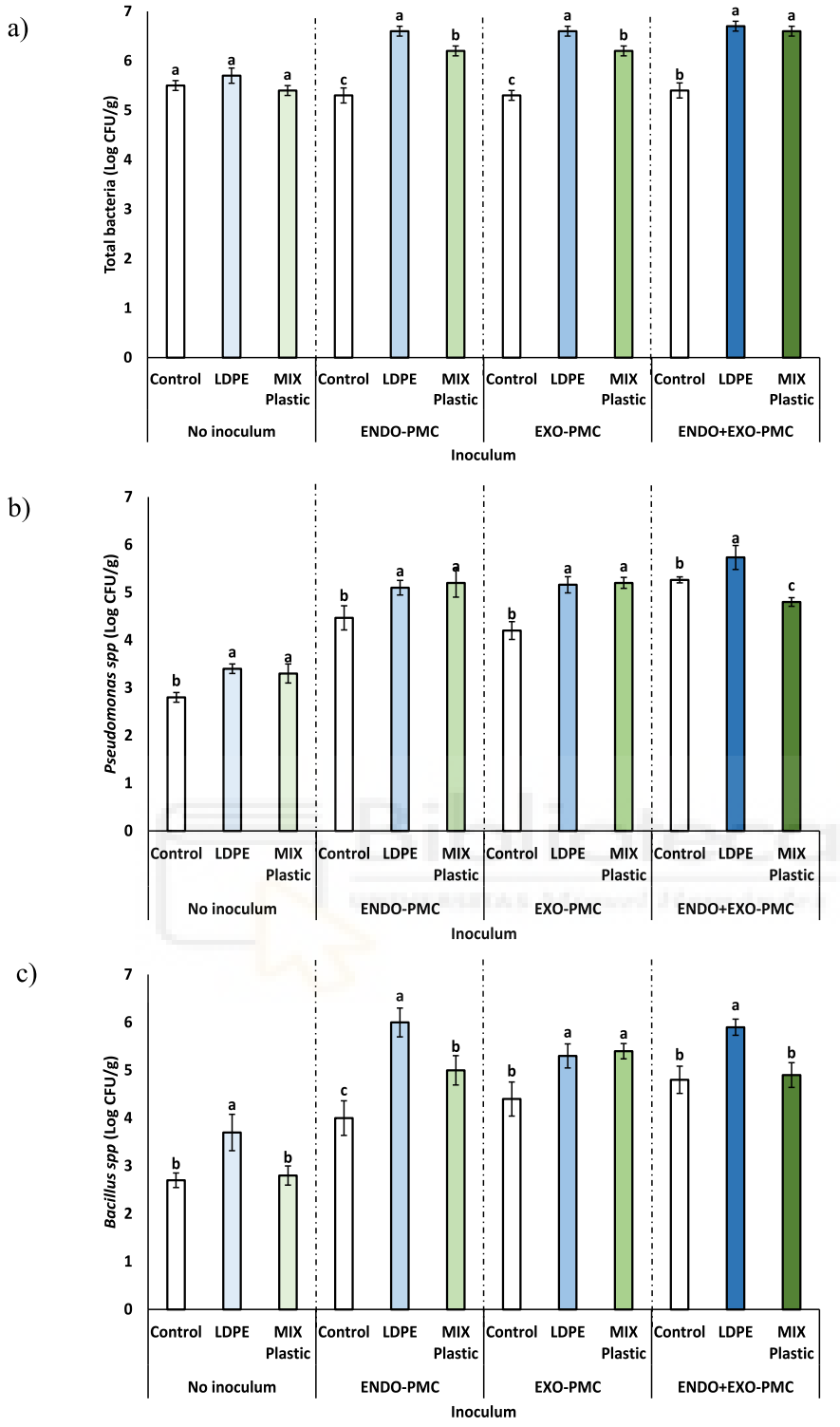


Figure 3. Logarithm of colony forming units (Log CFU g - 1) of the digestive tract of *E.fetida* obtained after PMC (ENDO,EXO and Mix C ENDO+EXO-PMC) inoculation in plastic spiked (LDPE and MIX Plastic) feedstock No inoculum and no plastic controls were set as negative controls for PMC inoculation and plastic presence,respectively. The results represent the means (n = 3) ± SD (vertical bars). The bars illustrate the total counts of bacteria (a); *Pseudomonas spp* (b); and *Bacillus spp* (c) in each experimental condition tested.

In order to trace the presence and load of inoculum in the vermicompost obtained, at the end of bioassay metataxonomic characterisation was also done in vermicompost for each experimental conditions tested (Fig 4). The inoculation with the probiotics had a clear impact on increase of *Pseudomonas* and *Bacillus spp*, especially when vermicompost were exposed to plastic materials. The results validated the successful colonization of the vermicompost by two type of consortium bacteria after 30 days, which increased in all sample with respect to initial load inoculated. Also, the fact that the levels for the two microorganisms where much higher in the inoculated than in the non-inoculated vermicompost, of the order of 2 log units and 1.5 log units for *Pseudomonas* and *Bacillus spp*, respectively. This increment in count of colony forming units in inoculated sample with respect to no inoculated could indicate that this load increase come from the microorganisms inoculated and not from indigenous *Pseudomonas* or *Bacillus* in Initial vermicompost.

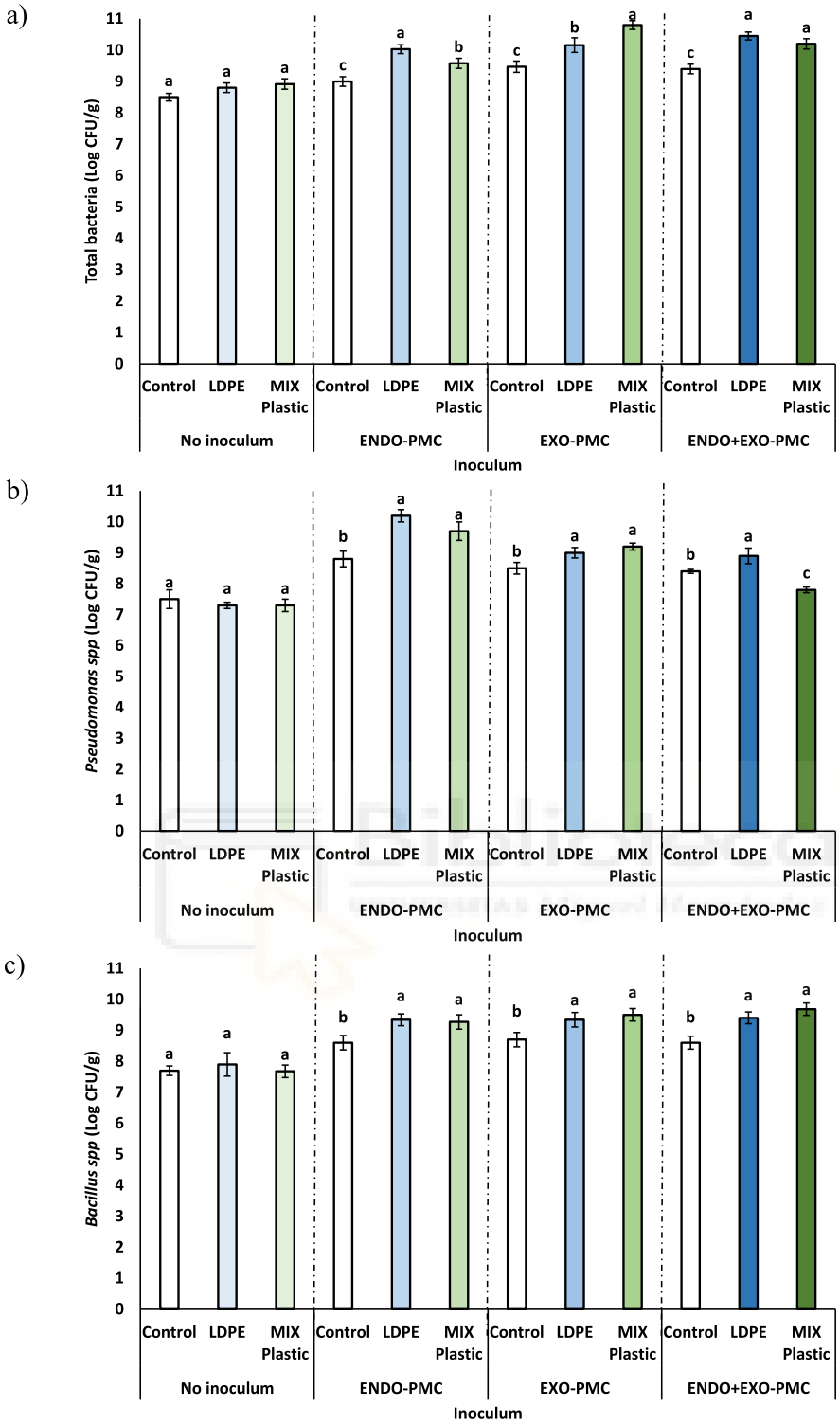


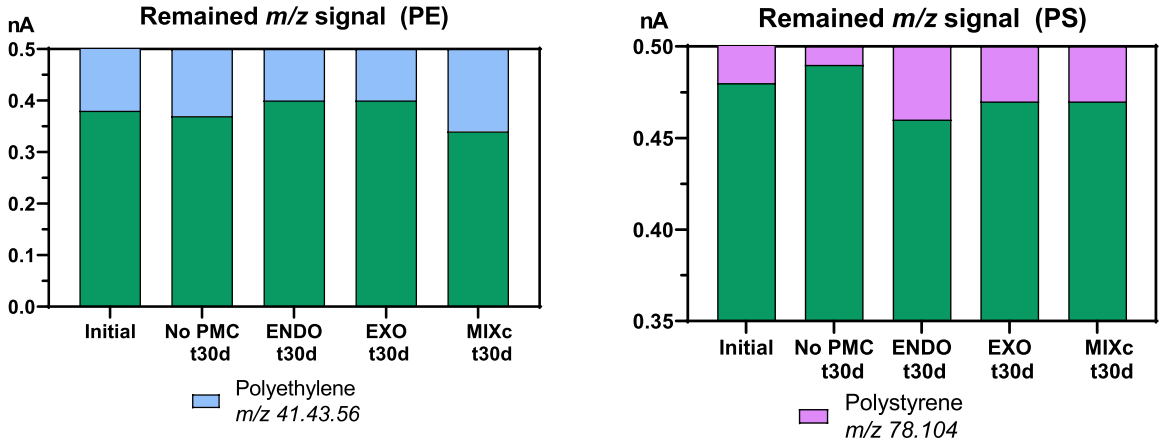
Figure 4. Logarithm of colony forming units (Log CFU g - 1) of the plastic spiked biowaste (LDPE and MIX Plastic) obtained after PMC (ENDO,EXO and Mix C ENDO+EXO-PMC) inoculation. No inoculum and no plastic controls were set as negative controls for PMC inoculation and plastic presence,respectively. The results represent means (n = 3) ± SD (vertical bars). Bars illustrate the total bacterial counts (a); *Pseudomonas spp* (b); *and Bacillus spp* (c) in each experimental condition tested. Homogeneity groups are represented respecting to each inoculum.

3.7 Biodegradation performance of PE and PS in PMC-inoculated bioassay

The *m/z* ions determined by TGA-MS could be grouped for each type of plastic, from PE the *m/z* 41,43 and 56 ions produce the highest intensity values. These ions corresponding to C_3H_5, C_3H_7, and C_4H_8, respectively and have been related with thermal degradation products of PE (Dümichen et al., 2015). On the other hand, the highest signals for PS corresponding with *m/z* 78 and 104, related to benzene (C_6H_6) and styrene (C_8H_8), respectively (Qureshi et al.,2020). In our study, the Initial (t 0d) and final control samples (un-spiked organic samples) were also analysed and compared to eliminate interferences from the natural evolution of the organic sample matrix. Regarding to control treatment without plastic added an increase in MS signal intensity was noticed for *m/z* 78 (13 %), while minimal change was found for *m/z* 41,43,56,104 with ±2 % of signal change at the end of bioassay if compared with initial sample. It is worth noting that the same behaviour was found in ENDO and EXO sample without plastic added, while in MIX c without plastic, observed an increase after of 30 days of vermicomposting, especially in *m/z* 78 and 104, with mean signal increase of 50 %. This increased trend we assumed that is natural alteration of the functional group from evolution of organic matrix in presence of this type of microbial consortium.

After of 30 days of vermicomposting (Fig 5), for polyethylene spiked samples found a decrease in intensity of MS ions in ENDO and EXO with mean value of -21 and -22 %, respectively, which might be related to the degradation of MPs and alteration of the functional groups of plastic material (Sintim et al.,2019). The sample without PMC addition (No PMC) not showed changes if compared with Initial sample. In opposite trend, MIX c noticed an increase in signals of ions that provide information about PE, which could be related with the mass and volume loss inherent to organic matter degradation (Sáez et al.,2017). In the determination of ions related with PS, only a decrease in signal were found for No PMC treatment, whilst in EXO, MIX c and especially in ENDO (Fig 5), a higher intensity in ions measured were found. Therefore, it seems that the rate of degradation of organic matter and the loss of volume is quicker than the degradation process of PS. In agree with this finding, Sintim et al 2019 not found degradation sign in PE plastic after 18 weeks of composting. In contrast with the observed in our study, Ragoobuer et al.,2013 reported that vermicomposting contributed to the reduction (22–31%) and promoted the biodegradability of PP-MPs and HDPE-MPs. The abundance of PP HDPE, measured by FTIR technique decreased significantly by 31 %

158 and 22 % respectively after 14 weeks of vermicomposting (measured by FTIR technique). Based on the
159 result obtained, we assumed that TGA-MS technique can be used to quantify the abundance of PS into
160 organic matrix but not able to provide information about biodegradability process of this type of plastic.



161 ***Figure 5.*** Relative abundance of *m/z* signal obtained in MS analysis related to PE (*m/z* 41.43.56) and PS (*m/z* 78,104).
162

163 ***4. Conclusions***

164 According to the results obtained on quantification at the *Pseudomonas* and *Bacillus* genus, the ENDO-
165 PMC seem to colonize the gut-microbiome of *Eisenia*, especially when exposed to plastic. On the other
166 hand, the EXO-PMC inoculum could persist in vermicompost after 30 days and increase their load despite
167 the interferences of the indigenous microbiota of the same species. The successful colonization contributes
168 to fortify the capabilities of Earthworms as showed the reduction in mortality and improve in loss of body
169 weight. In addition, the application of EXO-PMC and ENDO-PMC showed a reduction in signs of stress
170 oxidative as revealed the lower values of A_i score of enzymatic biomarkers when compared with treatment
171 non inoculated. The consortium selected not did not cause significant difference in characteristics of
172 vermicompost obtained. Finally, after the 30 days of bioassay the complexity of organic matrix and the
173 natural evolution as vermcicompost process progressed did not allow establish a clear relationship between
174 the intensity of the ions (*m/z*) measured by TGA-MS technique and the biodegradation process.

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